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<b>(21) International Application Number:</b> PCT/US95/09058 <b>(22) International Filing Date:</b> 23 June 1995 (23.06.95)  <b>(30) Priority Data:</b> 08/446,881                      5 May 1995 (05.05.95)                      US  <b>(71) Applicant:</b> HUMAN GENOME SCIENCES, INC. [US/US]; 9410 Key West Avenue, Rockville, MD 20850-3338 (US).  <b>(72) Inventors:</b> ROSEN, Craig, A.; 22400 Rolling Hill Road, Laytonsville, MD 20882 (US). RUBEN, Steven, M.; 18528 Heritage Hills Drive, Olney, MD 20832 (US). LI, Haodong; 11033 Rutledge Drive, Gaithersburg, MD 20878 (US). ADAMS, Mark, D.; 15205 Dufief Drive, North Potomac, MD 20878 (US).  <b>(74) Agents:</b> GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).		<b>(81) Designated States:</b> AU, CA, CN, JP, KR, MX, NZ, SI, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> HUMAN CHEMOKINE BETA-8, CHEMOKINE BETA-1 AND MACROPHAGE INFLAMMATORY PROTEIN-4  <b>(57) Abstract</b>  Human Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 and DNA (RNA) encoding such chemokine polypeptides and a procedure for producing such polypeptides by recombinant techniques is disclosed. Also disclosed are methods for utilizing such chemokine polypeptides for the treatment of leukemia, tumors, chronic infections, autoimmune disease, fibrotic disorders, wound healing and psoriasis. Antagonists against such chemokine polypeptides and their use as a therapeutic to treat rheumatoid arthritis, autoimmune and chronic and acute inflammatory and infective diseases, allergic reactions, prostaglandin-independent fever and bone marrow failure are also disclosed. Also disclosed are diagnostic assays for detecting diseases related to mutations in the nucleic acid sequences and for detecting altered concentrations of the polypeptides.		

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**HUMAN CHEMOKINE BETA-8, CHEMOKINE BETA-1  
AND MACROPHAGE INFLAMMATORY PROTEIN-4**

This application is a continuation-in-part of pending application serial number 08/446,881 filed in the United States Patent and Trademark Office on May 5, 1995.

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. More particularly, the polypeptides of the present invention have been putatively identified as human Chemokine Beta-8 ( $\text{Ck}\beta$ -8), macrophage inflammatory protein-4 (MIP-4) and Chemokine Beta-1 ( $\text{Ck}\beta$ -1). The invention also relates to inhibiting the action of such polypeptides.

Chemokines, also referred to as intercrine cytokines, are a subfamily of structurally and functionally related cytokines. These molecules are 8-10 kd in size. In general, chemokines exhibit 20% to 75% homology at the amino acid level and are characterized by four conserved cysteine residues that form two disulfide bonds. Based on the arrangement of the first two cysteine residues, chemokines

have been classified into two subfamilies, alpha and beta. In the alpha subfamily, the first two cysteines are separated by one amino acid and hence are referred to as the "C-X-C" subfamily. In the beta subfamily, the two cysteines are in an adjacent position and are, therefore, referred to as the "C-C" subfamily. Thus far, at least eight different members of this family have been identified in humans.

The intercrine cytokines exhibit a wide variety of functions. A hallmark feature is their ability to elicit chemotactic migration of distinct cell types, including monocytes, neutrophils, T lymphocytes, basophils and fibroblasts. Many chemokines have proinflammatory activity and are involved in multiple steps during an inflammatory reaction. These activities include stimulation of histamine release, lysosomal enzyme and leukotriene release, increased adherence of target immune cells to endothelial cells, enhanced binding of complement proteins, induced expression of granulocyte adhesion molecules and complement receptors, and respiratory burst. In addition to their involvement in inflammation, certain chemokines have been shown to exhibit other activities. For example, macrophage inflammatory protein 1 (MIP-1) is able to suppress hematopoietic stem cell proliferation, platelet factor-4 (PF-4) is a potent inhibitor of endothelial cell growth, Interleukin-8 (IL-8) promotes proliferation of keratinocytes, and GRO is an autocrine growth factor for melanoma cells.

In light of the diverse biological activities, it is not surprising that chemokines have been implicated in a number of physiological and disease conditions, including lymphocyte trafficking, wound healing, hematopoietic regulation and immunological disorders such as allergy, asthma and arthritis. An example of a hematopoietic lineage regulator is MIP-1. MIP-1 was originally identified as an endotoxin-induced proinflammatory cytokine produced from macrophages. Subsequent studies have shown that MIP-1 is composed of two



different, but related, proteins MIP-1 $\alpha$  and MIP-1 $\beta$ . Both MIP-1 $\alpha$  and MIP-1 $\beta$  are chemo-attractants for macrophages, monocytes and T lymphocytes. Interestingly, biochemical purification and subsequent sequence analysis of a multi-potent stem cell inhibitor (SCI) revealed that SCI is identical to MIP-1 $\alpha$ . Furthermore, it has been shown that MIP-1 $\beta$  can counteract the ability of MIP-1 $\alpha$  to suppress hematopoietic stem cell proliferation. This finding leads to the hypothesis that the primary physiological role of MIP-1 is to regulate hematopoiesis in bone marrow, and that the proposed inflammatory function is secondary. The mode of action of MIP-1 $\alpha$  as a stem cell inhibitor relates to its ability to block the cell cycle at the G1/S interphase. Furthermore, the inhibitory effect of MIP-1 $\alpha$  seems to be restricted to immature progenitor cells and it is actually stimulatory to late progenitors in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF).

Several groups have cloned what are likely to be the human homologs of MIP-1 $\alpha$  and MIP-1 $\beta$ . In all cases, cDNAs were isolated from libraries prepared against activated T-cell RNA.

MIP-1 proteins can be detected in early wound inflammation cells and have been shown to induce production of IL-1 and IL-6 from wound fibroblast cells. In addition, purified native MIP-1 (comprising MIP-1, MIP-1 $\alpha$  and MIP-1 $\beta$  polypeptides) causes acute inflammation when injected either subcutaneously into the footpads of mice or intracisternally into the cerebrospinal fluid of rabbits (Wolpe and Cerami, 1989, FASEB J. 3:2565-73). In addition to these pro-inflammatory properties of MIP-1, which may be direct or indirect, MIP-1 has been recovered during the early inflammatory phases of wound healing in an experimental mouse model employing sterile wound chambers (Fahey, et al., 1990, Cytokine, 2:92). For example, PCT application WO 92/05198, filed by Chiron Corporation, discloses a DNA molecule which

is active as a template for producing mammalian macrophage inflammatory proteins (MIPs) in yeast.

The murine MIP-1 $\alpha$  and MIP-1 $\beta$  are distinct but closely related cytokines. Partially purified mixtures of the two proteins affect neutrophil function and cause local inflammation and fever. MIP-1 $\alpha$  has been expressed in yeast cells and purified to homogeneity. Structural analysis confirmed that MIP-1 $\alpha$  has a very similar secondary and tertiary structure to PF-4 and IL-8 with which it shares limited sequence homology. It has also been demonstrated that MIP-1 $\alpha$  is active in vivo to protect mouse stem cells from subsequent in vitro killing by tritiated thymidine. MIP-1 $\alpha$  was also shown to enhance the proliferation of more committed progenitor granulocyte macrophage colony-forming cells in response to granulocyte macrophage colony-stimulating factor (Clemens, J.M., et al., Cytokine, 4:76-82 (1992)).

The polypeptides of the present invention, Ck $\beta$ -1, originally referred to as MIP-1 $\gamma$  in the parent patent application, is a new member of the  $\beta$  chemokine family based on amino sequence homology. The Ck $\beta$ -8 polypeptide, originally referred to as MIP-3 in the parent application, is also a new member of the  $\beta$  chemokine family based on the amino acid sequence homology.

In accordance with one aspect of the present invention, there are provided novel mature polypeptides which are human Ck $\beta$ -8, human MIP-4 and human Ck $\beta$ -1 as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding such polypeptides, including mRNAs, DNAs, cDNAs, genomic DNA as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques which comprises culturing recombinant prokaryotic and/or eukaryotic host cells, containing nucleic acid sequences, under conditions promoting expression of said proteins and subsequent recovery of said proteins.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides for therapeutic purposes, for example, to protect bone marrow stem cells from chemotherapeutic agents during chemotherapy, to remove leukemic cells, to stimulate an immune response, to regulate hematopoiesis and lymphocyte trafficking, to treat psoriasis, solid tumors, to enhance host defenses against resistant chronic and acute infection, and to stimulate wound healing.

In accordance with yet a further aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, to inhibit production of IL-1 and TNF- $\alpha$ , to treat aplastic anemia, myelodysplastic syndrome, asthma and arthritis.

In accordance with yet another aspect of the present invention, there are also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to the Ck $\beta$ -8, Ck $\beta$ -1 and MIP-4 nucleic acid sequences.

In accordance with still another aspect of the present invention, there are provided diagnostic assays for detecting diseases related to the underexpression and overexpression of

the polypeptides and for detecting mutations in the nucleic acid sequences encoding such polypeptides.

In accordance with yet another aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, as research reagents for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, for the purpose of developing therapeutics and diagnostics for the treatment of human disease.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

FIG. 1 displays the cDNA sequence encoding Ck $\beta$ -8 and the corresponding deduced amino acid sequence. The initial 21 amino acids represents the putative leader sequence. All the signal sequences were as determined by N-terminal peptide sequencing of the baculovirus expressed protein.

FIG. 2 displays the cDNA sequence encoding Ck $\beta$ -1 and the corresponding deduced amino acid sequence. The initial 19 amino acids represent the leader sequence.

FIG. 3 displays the cDNA sequence encoding MIP-4 and the corresponding deduced amino acid sequence. The initial 20 amino acids represent the leader sequence.

FIG. 4 illustrates the amino acid homology between Ck $\beta$ -8 (top) and human MIP-1 $\alpha$  (bottom). The four cysteines characteristic of all chemokines are shown.

FIG. 5 displays two amino acid sequences wherein, the top sequence is the human MIP-4 amino acid sequence and the bottom sequence is human MIP-1 $\alpha$  (Human Tonsillar lymphocyte LD78 Beta protein precursor).

FIG. 6 illustrates the amino acid sequence alignment between Ck $\beta$ -1 (top) and human MIP-1 $\alpha$  (bottom).

FIG. 7 is a photograph of a gel in which Ck $\beta$ -1 has been electrophoresed after the expression of HA-tagged Ck $\beta$ -1 in COS cells.

FIG. 8 is a photograph of a SDS-PAGE gel after expression and purification of Ck $\beta$ -1 in a baculovirus expression system.

FIG. 9 is a photograph of an SDS-PAGE gel after expression and a three-step purification of Ck $\beta$ -8 in a baculovirus expression system.

FIG. 10. The chemoattractant activity of Ck $\beta$ -8 was determined with chemotaxis assays using a 48-well microchamber device (Neuro Probe, Inc.). The experimental procedure was as described in the manufacturers manual. For each concentration of Ck $\beta$ -8 tested, migration in 5 high-power fields was examined. The results presented represent the average values obtained from two independent experiments. The chemoattractant activity on THP-1 (A) cells and human PBMCs (B) is shown.

FIG. 11. Change in intracellular calcium concentration in response to Ck $\beta$ -8 was determined using a Hitachi F-2000 fluorescence spectrophotometer. Bacterial expressed Ck $\beta$ -8 was added to Indo-1 loaded THP-1 cells to a final concentration of 50 nM and the intracellular level of calcium concentration was monitored.

FIG. 12. The monocyte cell line THP-1 was treated for 16 hours with LPS (0.1-10 ng/ml) or Ck $\beta$ -8 (to 50 ng/ml). Tissue culture supernatants were subjected to ELISA analysis to quantify the secretion of TNF- $\alpha$ .

FIG. 13. Human peripheral blood monocytes purified by elutriation were treated for 16 hours with increasing amounts of Ck $\beta$ -8 (produced by baculovirus). Tissue culture supernatants were subjected to ELISA analysis to quantify the secretion of TNF- $\alpha$ , IL-6, IL-1, GM-CSF, and granulocyte-colony stimulating factor (G-CSF).

FIG. 14. A low density population of mouse bone marrow cells was plated (1,500 cells/dish) in agar containing medium with or without the indicated chemokines (100 ng/ml), but in the presence of IL-3 (5 ng/ml), SCF (100 ng/ml), IL-1 $\alpha$  (10 ng/ml), and M-CSF (5 ng/ml). The data shown represents the average obtained from two independent experiments (each performed in duplicate). Colonies were counted 14 days after plating. The number of colonies generated in the presence of chemokines is expressed as a mean percentage of those produced in the absence of any added chemokines.

FIG. 15 illustrates the effect of Ck $\beta$ -8 and Ck $\beta$ -1 on mouse bone marrow colony formation by HPP-CFC (A) and LPP-CFC (B).

FIG. 16 illustrates the effect of baculovirus-expressed Ck $\beta$ -1 and Ck $\beta$ -8 on M-CFS and SCF-stimulated colony formation of freshly isolated bone marrow cells.

FIG. 17 illustrates the effect of Ck $\beta$ -8 and Ck $\beta$ -1 on IL-3 and SCF-stimulated proliferation and differentiation of the lin<sup>+</sup> population of bone marrow cells.

FIG. 18. Effect of Ck $\beta$ -8 and Ck $\beta$ -1 on the generation of GR-1 and Mac-1 (surface markers) positive population of cells from lin<sup>+</sup> population of bone marrow cells. lin<sup>+</sup> cells were incubated in growth medium supplemented with IL-3 (5 ng/ml) and SCF (100 ng/ml) alone (a) and Ck $\beta$ -8 (50 ng/ml) (b) or Ck $\beta$ -1 (50 ng/ml). Cells were then stained with Monoclonal antibodies against myeloid differentiation GR.1, Mac-1, Sca-1, and CD45R surface antigens and analyzed by FACScan. Data is presented as percentage of positive cells in both large (A) and small (B) cell populations.

FIG. 19 illustrates that the presence of Ck $\beta$ -8 (+) inhibits bone marrow cell colony formation in response to IL-3, M-CSF and GM-CSF.

FIG. 20. Dose response of Ck $\beta$ -8 inhibits bone marrow cell colony formation. Cells were isolated and treated as in Figure 19. The treated cells were plated at a density of

1,000 cells/dish in agar-based colony formation assays in the presence of IL-3, GM-CSF or M-CSF (5 ng/ml) with or without Ck $\beta$ -8 at 1, 10, 50 and 100 ng/ml. The data is presented as colony formation as a percentage of the number of colonies formed with the specific factor alone. The data is depicted as the average of duplicate dishes with error bars indicating the standard deviation.

FIG. 21. Induction of apoptosis by Ck $\beta$ -8 and Ck $\beta$ -1 in the presence or absence of hematopoietic growth factors. Mouse bone marrow cells were flushed from both the femur and tibia, separated on a ficol density gradient and monocytes removed by plastic adherence. The resulting population of cells were then incubated overnight in an MEM-based medium supplemented with IL-3 (5 ng/ml), GM-CSF (5 ng/ml), M-CSF (10 ng/ml) and G-CSF (10 ng/ml) with or without the addition of Ck $\beta$ -8 (50 ng/ml) or Ck $\beta$ -1 (250 ng/ml). In addition, cells were cultured in medium alone, with or without Ck $\beta$ -8 and Ck $\beta$ -1. After 24 hours, cells were harvested and processed for apoptosis using the boehringer mannheim cell death ELISA kit. Data is shown as percentage increase above background with the background considered as the amount of apoptosis occurring in the cultures incubated in the presence of each of the growth factors.

FIG. 22. Expression of RNA encoding Ck $\beta$ -8 in human monocytes. Total RNA from fresh elutriated monocytes was isolated and treated with 100 U/ml hu rIFN-g. 100 ng/ml LPS, or both. RNA (8  $\mu$ g) from each treatment was separated electrophoretically on a 1.2% agarose gel and transferred to a nylon membrane. Ck $\beta$ -8 mRNA was quantified by probing with <sup>32</sup>P-labeled cDNA and the bands on the resulting autoradiograph were quantified densitometrically.

In accordance with an aspect of the present invention, there are provided isolated nucleic acids (polynucleotides) which encode for the mature polypeptides having the deduced amino acid sequence of Figures 1, 2 and 3 (SEQ ID No. 2, 4

and 6, respectively) or for the mature Ck $\beta$ -8 polypeptide encoded by the cDNA of the clone(s) deposited as ATCC Deposit No. 75676 on February 9, 1994, and for the mature MIP-4 polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75675 on February 9, 1994 and for the mature Ck $\beta$ -1 polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75572, deposited on October 13, 1993.

Polynucleotides encoding polypeptides of the present invention are structurally related to the pro-inflammatory supergene "intercrine" which is in the cytokine or chemokine family. Both Ck $\beta$ -8 and MIP-4 are MIP-1 homologues and are more homologous to MIP-1 $\alpha$  than to MIP-1 $\beta$ . The polynucleotide encoding for Ck $\beta$ -8 was derived from an aortic endothelium cDNA library and contains an open reading frame encoding a polypeptide of 120 amino acid residues, which exhibits significant homology to a number of chemokines. The top match is to the human macrophage inflammatory protein 1 alpha, showing 36% identity and 66% similarity (figure 4).

The polynucleotide encoding for MIP-4 was derived from a human adult lung cDNA library and contains an open reading frame encoding a polypeptide of 89 amino acid residues, which exhibits significant homology to a number of chemokines. The top match is to the human tonsillar lymphocyte LD78 beta protein, showing 60% identity and 89% similarity (figure 5). Furthermore, the four cysteine residues occurring in all chemokines in a characteristic motif are conserved in both clone(s). The fact that the first two cysteine residues in the genes are in adjacent positions classifies them as "C-C" or  $\beta$  subfamily of chemokines. In the other subfamily, the "CXC" or  $\alpha$  subfamily, the first two cysteine residues are separated by one amino acid.

The polynucleotide encoding from Ck $\beta$ -1 contains an open reading frame encoding a polypeptide of 93 amino acids of which the first 19 are a leader sequence such that the mature polypeptide contains 74 amino acid residues. Ck $\beta$ -1 exhibits



significant homology to human macrophage inflammatory protein  $\alpha$  with 48% identity and 72% similarity over a stretch of 80 amino acids. Further, the four cysteine residues comprising a characteristic motif are conserved.

The polynucleotides of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptides may be identical to the coding sequence shown in Figures 1, 2 and 3 (SEQ ID No. 1, 3 and 5) or that of the deposited clone(s) or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same, mature polypeptides as the DNA of Figure 1, 2 and 3 (SEQ ID No. 1, 3 and 5) or the deposited cDNA(s).

The polynucleotides which encode for the mature polypeptides of Figures 1, 2 and 3 (SEQ ID No. 2, 4 and 6) or for the mature polypeptides encoded by the deposited cDNA(s) may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptides and additional coding sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptides (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptides.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figures 1, 2 and 3 (SEQ ID

No. 2, 4 and 6) or the polypeptides encoded by the cDNA of the deposited clone(s). The variants of the polynucleotides may be a naturally occurring allelic variant of the polynucleotides or a non-naturally occurring variant of the polynucleotides.

Thus, the present invention includes polynucleotides encoding the same mature polypeptides as shown in Figures 1, 2 and 3 (SEQ ID No. 2, 4 and 6) or the same mature polypeptides encoded by the cDNA of the deposited clone(s) as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptides of Figures 1, 2 and 3 (SEQ ID No. 2, 4 and 6) or the polypeptides encoded by the cDNA of the deposited clone(s). Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figures 1, 2 and 3 (SEQ ID No. 1, 3 and 5) or of the coding sequence of the deposited clone(s). As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptides may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode

for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotides of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptides of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptides fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 50% and preferably 70% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which retain substantially the same biological function or activity as the mature polypeptides encoded by the cDNA of Figure 1, 2 and 3 (SEQ ID No. 1, 3 and 5) or the deposited cDNAs.

Alternatively, the polynucleotides may be polynucleotides which has at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which does not retain activity. Such polynucleotides may be employed as probes for the polynucleotides of SEQ ID NOS:1, 3 and 5 for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 polypeptides which have the deduced amino acid sequence of Figures 1, 2 and 3 (SEQ ID No. 2, 4 and 6) or which have the amino acid sequence encoded by the deposited cDNAs, as well as fragments, analogs and derivatives of such polypeptides.

The terms "fragment," "derivative" and "analog" when referring to the polypeptides of Figures 1, 2 and 3 (SEQ ID No. 2, 4 and 6) or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of

the proprotein portion to produce an active mature polypeptide.

The polypeptides of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptides of Figures 1, 2 and 3 (SEQ ID No. 2, 4 and 6) or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptides are fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptides, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptides or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "gene" or "cistron" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotides or polypeptides present in a living

animal is not isolated, but the same polynucleotides or DNA or polypeptides, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the  $Ck\beta$ -8, MIP-4 and  $Ck\beta$ -1 genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide sequence may be included in any one of a variety of expression vehicles, in particular vectors or plasmids for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other plasmid or

vector may be used as long they are replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda P<sub>L</sub> promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella Typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Sf9; adenoviruses; animal cells such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate

host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); pTRC99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P<sub>R</sub>, P<sub>L</sub> and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast



cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct

transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the

structural sequence to be expressed. F o l l o w i n g transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 are recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used,

as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The polypeptides of the present invention may be employed in a variety of immunoregulatory and inflammatory functions and also in a number of disease conditions.  $\text{Ck}\beta$ -8, MIP-4 and  $\text{Ck}\beta$ -1 are in the chemokine family and therefore they are chemoattractants for leukocytes (such as monocytes, neutrophils, T lymphocytes, eosinophils, basophils, etc.).

Northern Blot analyses show that  $\text{Ck}\beta$ -8, MIP-4 and  $\text{Ck}\beta$ -1 are expressed predominantly in tissues of haemopoietic origin.

$\text{Ck}\beta$ -8 is shown to play an important role in the regulation of the immune response and inflammation. In Figure 22, it is shown that lipopolysaccharide induces the expression of  $\text{Ck}\beta$ -8 from human monocytes. Accordingly, in response to the presence of an endotoxin,  $\text{Ck}\beta$ -8 is expressed from monocytes and, therefore, administration of  $\text{Ck}\beta$ -8 may be employed to regulate the immune response of a host.

As illustrated in Figure 10, the chemoattractant activity of  $\text{Ck}\beta$ -8 on THP-1 cells (A) and PBMCs (B) is significant.  $\text{Ck}\beta$ -8 also induces significant calcium mobilization in THP-1 cells (Figure 11) showing that  $\text{Ck}\beta$ -8 has a biological effect on monocytes. Further,  $\text{Ck}\beta$ -8

produces a dose dependent chemotactic and calcium mobilization response in human monocytes.

Accordingly, Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 can be employed to facilitate wound healing by controlling infiltration of target immune cells to the wound area. In a similar fashion, the polypeptides of the present invention can enhance host defenses against chronic infections, e.g., mycobacterial, via their attraction and activation of microbicidal leukocytes.

Further, the polypeptides of the present invention may be employed in anti-tumor therapy since there is evidence that chemokine expressing cells injected into tumors have caused regression of the tumor, for example, in the treatment of Kaposi sarcoma. An analysis of Figures 12 and 13 illustrate that Ck $\beta$ -8 induces THP-1 cells to secrete TNF- $\alpha$ , which is a known agent for regressing tumors. 250 ng/ml of Ck $\beta$ -8 induces the production and secretion of 1200 picograms/ml of TNF- $\alpha$ . Ck $\beta$ -8 also significantly induces human monocytes to secrete other tumor and cancer inhibiting agents such as IL-6, IL-1 and G-CSF. Also, Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 stimulate the invasion and activation of host defense (tumoricidal) cells, e.g., cytotoxic T-cells and macrophages via their chemotactic activity, and in this way may also be employed to treat solid tumors.

The polypeptides may also be employed to inhibit the proliferation and differentiation of hematopoietic cells and therefore may be employed to protect bone marrow stem cells from chemotherapeutic agents during chemotherapy. Figures 14 and 15 illustrate that Ck $\beta$ -8 and Ck $\beta$ -1 inhibit colony formation of low proliferative potential colony forming cells, and that Ck $\beta$ -8 is a potent and specific inhibitor of LPP-CFC colony growth. Figure 16 illustrates that Ck $\beta$ -1 specifically inhibits M-CSF-stimulated colony formation, while Ck $\beta$ -8 does not. However, as also shown, both Ck $\beta$ -8 and Ck $\beta$ -1 significantly inhibit growth or differentiation of bone marrow cells. This antiproliferative effect allows a greater

exposure to chemotherapeutic agents and, therefore, more effective chemotherapeutic treatment.

The inhibitory effect of the  $Ck\beta$ -1 and  $Ck\beta$ -8 polypeptides on the subpopulation of committed progenitor cells, (for example granulocyte, and macrophage/monocyte cells) may be employed therapeutically to inhibit proliferation of leukemic cells.

In Figures 17, 18 and 19 the committed cells of the cell lineages utilized were removed and the resulting population of cells were contacted with  $Ck\beta$ -1 and  $Ck\beta$ -8.  $Ck\beta$ -1 causes a decrease in the Mac-1 positive population of cells by nearly 50%, which is consistent with the results of Figure 16 which shows  $Ck\beta$ -1 induces inhibition of M-CSF responsive colony-forming cells.  $Ck\beta$ -8, as shown in Figure 19, inhibits the ability of committed progenitor cells to form colonies in response to IL-3, GM-CSF and M-CSF. Further, as shown in Figure 20, a dose response of  $Ck\beta$ -8 is shown to inhibit colony formation. This inhibition could be due to a specific blockage of the differentiative signal mediated by these factors or to a cytotoxic effect on the progenitor cells.

Another employment of the polypeptides is the inhibition of T-cell proliferation via inhibition of IL-2 biosynthesis, for example, in auto-immune diseases and lymphocytic leukemia.

$Ck\beta$ -8, MIP-4 and  $Ck\beta$ -1 may also be employed for inhibiting epidermal keratinocyte proliferation for psoriasis (keratinocyte hyper-proliferation) since Langerhans cells in skin have been found to produce MIP-1 $\alpha$ .

$Ck\beta$ -8, MIP-4 and  $Ck\beta$ -1 may be employed to prevent scarring during wound healing both via the recruitment of debris-cleaning and connective tissue-promoting inflammatory cells and by control of excessive TGF $\beta$ -mediated fibrosis. In addition, these polypeptides may be employed to treat stroke, thrombocytosis, pulmonary emboli and myeloproliferative

disorders, since Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 increase vascular permeability.

Ck $\beta$ -8 may also be employed to treat leukemia and abnormally proliferating cells, for example tumor cells, by inducing apoptosis. Ck $\beta$ -8 induces apoptosis in a population of hematopoietic progenitor cells as shown in Figure 21.

The polypeptides of the present invention, and polynucleotides encoding such polypeptides, may be employed as research reagents for *in vitro* purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, and for the purpose of developing therapeutics and diagnostics for the treatment of human disease. For example, Ck $\beta$ -1 and Ck $\beta$ -8 may be employed for the expansion of immature hematopoietic progenitor cells, for example, granulocytes, macrophages or monocytes, by temporarily preventing their differentiation. These bone marrow cells may be cultured *in vitro*.

Fragments of the full length Ck $\beta$ -8, MIP-4 or Ck $\beta$ -1 genes may be used as a hybridization probe for a cDNA library to isolate the full length gene and to isolate other genes which have a high sequence similarity to the gene or similar biological activity. Preferably, however, the probes have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete genes including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the genes by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the genes of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

This invention is also related to the use of the Ck $\beta$ -8, MIP-4 and Ck $\alpha$ -1 gene as part of a diagnostic assay for detecting diseases or susceptibility to diseases related to the presence of mutations in the nucleic acid sequences. Such diseases are related to under-expression of the chemokine polypeptides.

Individuals carrying mutations in the Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., Nature, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid encoding Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 can be used to identify and analyze Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 RNA or alternatively, radiolabeled Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial



melting temperatures (see, e.g., Myers et al., Science, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton et al., PNAS, USA, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., Restriction Fragment Length Polymorphisms (RFLP)) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

The present invention also relates to a diagnostic assay for detecting altered levels of Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 protein in various tissues since an over-expression of the proteins compared to normal control tissue samples may detect the presence of a disease or susceptibility to a disease, for example, a tumor. Assays used to detect levels of Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 protein in a sample derived from a host are well-known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western Blot analysis, ELISA assays and "sandwich" assay. An ELISA assay (Coligan, et al., Current Protocols in Immunology, 1(2), Chapter 6, (1991)) initially comprises preparing an antibody specific to the Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 antigens, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or, in this example, a horseradish peroxidase enzyme. A sample is removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by

incubating with a non-specific protein like BSA. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 protein present in a given volume of patient sample when compared against a standard curve.

A competition assay may be employed wherein antibodies specific to Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 are attached to a solid support and labeled Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 and a sample derived from the host are passed over the solid support and the amount of label detected, for example by liquid scintillation chromatography, can be correlated to a quantity of protein in the sample.

A "sandwich" assay is similar to an ELISA assay. In a "sandwich" assay Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 is passed over a solid support and binds to antibody attached to a solid support. A second antibody is then bound to the Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1. A third antibody which is labeled and specific to the second antibody is then passed over the solid support and binds to the second antibody and an amount can then be quantified.

This invention provides a method for identification of the receptors for the chemokine polypeptides. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression

cloning is employed wherein polyadenylated RNA is prepared from a cell responsive to the polypeptides, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the polypeptides. Transfected cells which are grown on glass slides are exposed to the labeled polypeptides. The polypeptides can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clones that encodes the putative receptor.

As an alternative approach for receptor identification, the labeled polypeptides can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE analysis and exposed to X-ray film. The labeled complex containing the receptors of the polypeptides can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

This invention provides a method of screening compounds to identify agonists and antagonists to the chemokine polypeptides of the present invention. An agonist is a compound which has similar biological functions of the polypeptides, while antagonists block such functions. Chemotaxis may be assayed by placing cells, which are chemoattracted by either of the polypeptides of the present invention, on top of a filter with pores of sufficient diameter to admit the cells (about 5  $\mu$ m). Solutions of potential agonists are placed in the bottom of the chamber

with an appropriate control medium in the upper compartment, and thus a concentration gradient of the agonist is measured by counting cells that migrate into or through the porous membrane over time.

When assaying for antagonists, the chemokine polypeptides of the present invention are placed in the bottom chamber and the potential antagonist is added to determine if chemotaxis of the cells is prevented.

Alternatively, a mammalian cell or membrane preparation expressing the receptors of the polypeptides would be incubated with a labeled chemokine polypeptide, eg. radioactivity, in the presence of the compound. The ability of the compound to block this interaction could then be measured. When assaying for agonists in this fashion, the chemokines would be absent and the ability of the agonist itself to interact with the receptor could be measured.

Examples of potential Ck $\beta$ -8, MIP-4 and Ck $\beta$ -1 antagonists include antibodies, or in some cases, oligonucleotides, which bind to the polypeptides. Another example of a potential antagonist is a negative dominant mutant of the polypeptides. Negative dominant mutants are polypeptides which bind to the receptor of the wild-type polypeptide, but fail to retain biological activity.

Antisense constructs prepared using antisense technology are also potential antagonists. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix, see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et

al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of the chemokine polypeptides. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the polypeptides (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the chemokine polypeptides.

Another potential chemokine antagonist is a peptide derivative of the polypeptides which are naturally or synthetically modified analogs of the polypeptides that have lost biological function yet still recognize and bind to the receptors of the polypeptides to thereby effectively block the receptors. Examples of peptide derivatives include, but are not limited to, small peptides or peptide-like molecules.

The antagonists may be employed to treat disorders which are either MIP-induced or enhanced, for example, auto-immune and chronic inflammatory and infective diseases. Examples of auto-immune diseases include multiple sclerosis, and insulin-dependent diabetes.

The antagonists may also be employed to treat infectious diseases including silicosis, sarcoidosis, idiopathic pulmonary fibrosis by preventing the recruitment and activation of mononuclear phagocytes. They may also be employed to treat idiopathic hyper-eosinophilic syndrome by preventing eosinophil production and migration. Endotoxic shock may also be treated by the antagonists by preventing the migration of macrophages and their production of the chemokine polypeptides of the present invention.

The antagonists may also be employed for treating atherosclerosis, by preventing monocyte infiltration in the artery wall.

The antagonists may also be employed to treat histamine-mediated allergic reactions and immunological disorders including late phase allergic reactions, chronic urticaria, and atopic dermatitis by inhibiting chemokine-induced mast cell and basophil degranulation and release of histamine. IgE-mediated allergic reactions such as allergic asthma, rhinitis, and eczema may also be treated.

The antagonists may also be employed to treat chronic and acute inflammation by preventing the attraction of monocytes to a wound area. They may also be employed to regulate normal pulmonary macrophage populations, since chronic and acute inflammatory pulmonary diseases are associated with sequestration of mononuclear phagocytes in the lung.

Antagonists may also be employed to treat rheumatoid arthritis by preventing the attraction of monocytes into synovial fluid in the joints of patients. Monocyte influx and activation plays a significant role in the pathogenesis of both degenerative and inflammatory arthropathies.

The antagonists may be employed to interfere with the deleterious cascades attributed primarily to IL-1 and TNF, which prevents the biosynthesis of other inflammatory cytokines. In this way, the antagonists may be employed to prevent inflammation. The antagonists may also be employed to inhibit prostaglandin-independent fever induced by chemokines.

The antagonists may also be employed to treat cases of bone marrow failure, for example, aplastic anemia and myelodysplastic syndrome.

The antagonists may also be employed to treat asthma and allergy by preventing eosinophil accumulation in the lung. The antagonists may also be employed to treat subepithelial basement membrane fibrosis which is a prominent feature of the asthmatic lung.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The chemokine polypeptides and agonists and antagonists may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the polypeptides and agonists and antagonists may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, intratumor, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the polypeptides will be administered in an amount of at least about 10  $\mu\text{g/kg}$  body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10  $\mu\text{g/kg}$  to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The chemokine polypeptides, and agonists or antagonists which are polypeptides, may be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

The retroviral plasmid vectors may be derived from retroviruses which include, but are not limited to, Moloney Murine Sarcoma Virus, Moloney Murine Leukemia Virus, spleen necrosis virus, Rous Sarcoma Virus and Harvey Sarcoma Virus.

In a preferred embodiment the retroviral expression vector, pMV-7, is flanked by the long terminal repeats (LTRs) of the Moloney murine sarcoma virus and contains the selectable drug resistance gene neo under the regulation of the herpes simplex virus (HSV) thymidine kinase (tk)



promoter. Unique EcoRI and HindIII sites facilitate the introduction of coding sequence (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)).

The vectors include one or more suitable promoters which include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and  $\beta$ -actin promoters). The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter which includes, but is not limited to, viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs, the  $\beta$ -actin promoter, and the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317 and GP+am12. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO<sub>4</sub> precipitation.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced,

include but are not limited to, fibroblasts and endothelial cells.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNA to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of cDNA clones to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can

be used with cDNA as short as 500 or 600 bases. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention or its

in vivo receptor can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptides from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptides products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available,

publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5  $\mu$ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, P. and Van der Eb, A., Virology, 52:456-457 (1973).

#### Example 1

##### Bacterial Expression and Purification of Ck $\beta$ -8

The DNA sequence encoding Ck $\beta$ -8, ATCC # 75676, was initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' end sequences of the processed Ck $\beta$ -8 protein (minus the signal peptide sequence) and the vector sequences 3' to the Ck $\beta$ -8 gene. Additional nucleotides corresponding to Bam HI and XbaI were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' TCAGGATCCGTCACAAAAGATGCAGA 3' (SEQ ID No. 7) contains a BamHI restriction enzyme site followed by 18 nucleotides of Ck $\beta$ -8 coding sequence starting from the presumed terminal amino acid of the processed protein. The 3' sequence 5' CGCTCTAGAGTAAACGACGGCCAGT 3' (SEQ ID No. 8) contains complementary sequences to an XbaI site. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector PQE-9 (Qiagen, Inc., Chatsworth, CA). PQE-9 encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 is then digested with BamHI and XbaI. The amplified sequences are ligated into PQE-9 and are inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation

mixture is then used to transform E. coli strain M15/rep4 available from Qiagen. M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells are grown an extra 3 to 4 hours. Cells are then harvested by centrifugation. The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized Ck $\beta$ -8 is purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). Ck $\beta$ -8 (95% pure) is eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein is dialyzed to 10 mmolar sodium phosphate.

### Example 2

#### Bacterial Expression and Purification of MIP-4

The DNA sequence encoding MIP-4, ATCC # 75675, was initially amplified using PCR oligonucleotide primers

corresponding to the 5' and 3' sequences of the processed MIP-4 protein (minus the signal peptide sequence). Additional nucleotides corresponding to Bam HI and XbaI were added to the 5' and 3' end sequences respectively. The 5' oligonucleotide primer has the sequence 5' TCAGGATCCTGTGCACAAGTTGGTACC 3' (SEQ ID No. 9) contains a BamHI restriction enzyme site followed by 18 nucleotides of MIP-4 coding sequence starting from the presumed terminal amino acid of the processed protein codon; The 3' sequence 5' CGCTCTAGAGTAAAACGACGGCCAGT 3' (SEQ ID No. 10) contains complementary sequences to an XbaI site. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc., Chatsworth, CA). pQE-9 encodes antibiotic resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 was then digested with BamHI and XbaI and the amplified sequences were ligated into pQE-9 and inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform E. coli strain available from Qiagen. M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating



the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized MIP-4 was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). MIP-4 (95% pure) was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate.

### Example 3

#### Bacterial Expression and Purification of Ck $\beta$ -1

The DNA sequence encoding Ck $\beta$ -1, ATCC # 75572, is initially amplified using PCR oligonucleotide primers corresponding to the 5' and 3' end sequences of the processed Ck $\beta$ -1 protein (minus the signal peptide sequence) and additional nucleotides corresponding to Bam HI and XbaI were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' GCCGCGGATCCTCCTCACGGGGACCTTAC 3' (SEQ ID No. 11) contains a BamHI restriction enzyme site followed by 15 nucleotides of Ck $\beta$ -1 coding sequence starting from the presumed terminal amino acid of the processed protein codon; The 3' sequence 5' GCCTGCTCTAGATCAAAGCAGGGAAGCTCCAG 3' (SEQ ID No. 12) contains complementary sequences to an XbaI site, a translation stop codon and the last 20 nucleotides of Ck $\beta$ -1 coding sequence. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector PQE-9. (Qiagen, Inc., Chatsworth, CA). PQE-9 encodes antibiotic

resistance (Amp<sup>r</sup>), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 was then digested with BamHI and XbaI and the amplified sequences were ligated into pQE-9 and were inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture was then used to transform E. coli strain available from Qiagen under the trademark M15/rep 4. M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan<sup>r</sup>). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D.<sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation. The cell pellet was solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized Ck $\beta$ -1 was purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). Ck $\beta$ -1 (95% pure) was eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in

this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate.

#### Example 4

##### Expression of Recombinant Ck $\beta$ -8 in COS cells

The expression of plasmid, CMV-Ck $\beta$ -8 HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the entire Ck $\beta$ -8 precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag correspond to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, et al., Cell, 37:767 (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follow:

The DNA sequence encoding for Ck $\beta$ -8, ATCC # 75676, is constructed by PCR using two primers: the 5' primer 5' GGAAAGCTTATGAAGGTCTCCGTGGCT 3' (SEQ ID No. 13) contains a HindIII site followed by 18 nucleotides of Ck $\beta$ -8 coding sequence starting from the initiation codon; the 3' sequence 5' CGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAATTCTTCCTGGTCTTGATCC 3' (SEQ ID No. 14) contains complementary sequences to an Xba I site, translation stop codon, HA tag and the last 20 nucleotides of the Ck $\beta$ -8 coding sequence (not including the stop codon). Therefore, the PCR product contains a HindIII site, Ck $\beta$ -8 coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with HindIII and XbaI

restriction enzyme and ligated. The ligation mixture is transformed into *E. coli* strain SURE (Stratagene Cloning Systems, La Jolla, CA) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant Ck $\beta$ -8, COS cells are transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the Ck $\beta$ -8-HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media are then collected and cells are lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984))). Both cell lysate and culture media are precipitated with a HA specific monoclonal antibody. Proteins precipitated are analyzed on 15% SDS-PAGE gels.

#### Example 5

##### Expression of Recombinant MIP-4 in COS cells

The expression of plasmid, CMV-MIP-4 HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) *E. coli* replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the entire MIP-4 precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag correspond to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, et

al., Cell, 37:767 (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follow:

The DNA sequence encoding MIP-4, ATCC # 75675, is constructed by PCR using two primers: the 5' primer 5' GGAAAGCTTATGAAGGGCCTTGCAGCTGCC 3' (SEQ ID No. 15) contains a HindIII site followed by 20 nucleotides of MIP-4 coding sequence starting from the initiation codon; the 3' sequence 5' CGCTCTAGATCAABCGTAGTCTGGGACGTCGTATGGGTAGGCATTTCAGCTTCAGGTC 3' (SEQ ID No. 16) contains complementary sequences to an XbaI site, translation stop codon, HA tag and the last 19 nucleotides of the MIP-4 coding sequence (not including the stop codon). Therefore, the PCR product contains a HindIII site, MIP-4 coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with HindIII and XbaI restriction enzyme and ligated. The ligation mixture is transformed into E. coli strain SURE (Stratagene Cloning Systems, La Jolla, CA) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant MIP-4, COS cells are transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the MIP-4-HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media are then collected and cells are lysed with detergent (RIPA

buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media are precipitated with a HA specific monoclonal antibody. Proteins precipitated are analyzed on 15% SDS-PAGE gels.

#### Example 6

##### Expression of Recombinant Ck $\beta$ -1 in COS cells

The expression of plasmid, CMV-Ck $\beta$ -1 HA is derived from a vector pcDNA1/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, a SV40 intron and polyadenylation site. A DNA fragment encoding the entire Ck $\beta$ -1 precursor and a HA tag fused in frame to its 3' end was cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag correspond to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, et al., Cell, 37:767 (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding Ck $\beta$ -1, ATCC # 75572, was constructed by PCR using two primers: the 5' primer 5' GGAAAGCTTATGAAGATTCCGTGGCTGC 3' (SEQ ID No. 17) contains a HindIII site followed by 20 nucleotides of Ck $\beta$ -1 coding sequence starting from the initiation codon; the 3' sequence 5' CGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAGTTCTCCTTCATGTCCTTG 3' (SEQ ID No. 18) contains complementary sequences to an Xba I site, translation stop codon, HA tag and the last 19 nucleotides of the Ck $\beta$ -1 coding sequence (not including the stop codon). Therefore, the PCR product contains a HindIII site, Ck $\beta$ -1 coding sequence followed by an HA tag fused in

frame, a translation termination stop codon next to the HA tag, and an XbaI site. The PCR amplified DNA fragment and the vector, pCDNAI/Amp, were digested with HindIII and XbaI restriction enzyme and ligated. The ligation mixture was transformed into E. coli strain SURE (Stratagene Cloning Systems, La Jolla, CA) the transformed culture was plated on ampicillin media plates and resistant colonies were selected. Plasmid DNA was isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant Ck $\beta$ -1, COS cells were transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the Ck $\beta$ -1 HA protein was detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells were labelled for 8 hours with <sup>35</sup>S-cysteine two days post transfection. Culture media were then collected and cells were lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., *Id.* 37:767 (1984)). Both cell lysate and culture media were precipitated with a HA specific monoclonal antibody. Proteins precipitated were analyzed on 15% SDS-PAGE gels.

#### Example 7

##### Expression pattern of Ck $\beta$ -8 in human tissue

Northern blot analysis was carried out to examine the levels of expression of Ck $\beta$ -8 in human tissues. Total cellular RNA samples were isolated with RNAzol™ B system (Biotechx Laboratories, Inc., Houston, TX 77033). About 10ug of total RNA isolated from each human tissue specified is separated on 1% agarose gel and blotted onto a nylon filter (Sambrook, Fritsch, and Maniatis, Molecular Cloning, Cold Spring Harbor Press, (1989)). The labeling reaction is done

according to the Stratagene Prime-It kit with 50ng DNA fragment. The labeled DNA is purified with a Select-G-50 column. (5 Prime - 3 Prime, Inc. Boulder, CO). The filter is then hybridized with radioactive labeled full length Ck $\beta$ -8 gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub>, pH 7.4 and 7% SDS overnight at 65°C. After wash twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS, the filter is then exposed at -70°C overnight with an intensifying screen.

#### Example 8

##### Expression pattern of MIP-4 in human cells

Northern blot analysis was carried out to examine the levels of expression of MIP-4 in human cells. Total cellular RNA samples were isolated with RNazol™ B system (Biotech Laboratories, Inc., Houston, TX). About 10ug of total RNA isolated from each human tissue specified was separated on 1% agarose gel and blotted onto a nylon filter (Sambrook, Fritsch, and Maniatis, Molecular Cloning, Cold Spring Harbor Press, (1989)). The labeling reaction was done according to the Stratagene Prime-It kit with 50ng DNA fragment. The labeled DNA was purified with a Select-G-50 column. (5 Prime - 3 Prime, Inc., Boulder, CO). The filter was then hybridized with radioactive labeled full length MIP-4 gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub>, pH 7.4 and 7% SDS overnight at 65°C. After wash twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS, the filter was then exposed at -70°C overnight with an intensifying screen.

#### Example 9

##### Expression pattern of Ck $\beta$ -1 in human tissue

Northern blot analysis was carried out to examine the levels of expression of Ck $\beta$ -1 in human tissues. Total cellular RNA samples were isolated with RNazol™ B system (Biotech Laboratories, Inc. Houston, TX). About 10ug of total RNA isolated from each human tissue specified was



separated on 1% agarose gel and blotted onto a nylon filter (Sambrook, Fritsch, and Maniatis, Molecular Cloning, Cold Spring Harbor Press, (1989)). The labeling reaction was done according to the Stratagene Prime-It kit with 50ng DNA fragment. The labeled DNA was purified with a Select-G-50 column. (5 Prime - 3 Prime, Inc., Boulder, CO). The filter was then hybridized with radioactive labeled full length Ck $\beta$ -1 gene at 1,000,000 cpm/ml in 0.5 M NaPO<sub>4</sub>, pH 7.4 and 7% SDS overnight at 65°C. After wash twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS, the filter was then exposed at -70°C overnight with an intensifying screen. The message RNA for Ck $\beta$ -1 is abundant in spleen.

#### Example 10

##### Expression and Purification of Chemokine Ck $\beta$ -8 using a baculovirus expression system.

SP9 cells were infected with a recombinant baculovirus designed to express the Ck $\beta$ -8 cDNA. Cells were infected at an MOI of 2 and cultured at 28°C for 72-96 hours. Cellular debris from the infected culture was removed by low speed centrifugation. Protease inhibitor cocktail was added to the supernatant at a final concentration of 20  $\mu$ g/ml Pefabloc SC, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml E-64 and 1 mM EDTA. The level of Ck $\beta$ -8 in the supernatant was monitored by loading 20-30  $\mu$ l of supernatant only 15% SDS-PAGE gels. Ck $\beta$ -8 was detected as a visible 9 Kd band, corresponding to an expression level of several mg per liter. Ck $\beta$ -8 was further purified through a three-step purification procedure: Heparin binding affinity chromatography. Supernatant of baculovirus culture was mixed with 1/3 volume of buffer containing 100 mM HEPES/MES/NaOAc pH 6 and filtered through 0.22  $\mu$ m membrane. The sample was then applied to a heparin binding column (HE1 poros 20, Bio-Perceptive System Inc.). Ck $\beta$ -08 was eluted at approximately 300 mM NaCl in a linear gradient of 50 to 500 mM NaCl in 50 mM HEPES/MES/NaOAc at pH 6; Cation exchange chromatography.

The Ck $\beta$ 8 enriched from heparin chromatography was subjected to a 5-fold dilution with a buffer containing 50 mM HEPES/MES/NaOAc pH 6. The resultant mixture was then applied to a cation exchange column (S/M poros 20, Bio-Perceptive System Inc.). Ck $\beta$ -8 was eluted at 250 mM NaCl in a linear gradient of 25 to 300 mM NaCl in 50 mM HEPES/MES/NaOAc at pH 6; Size exclusion chromatography. Following the cation exchange chromatography, Ck $\beta$ -8 was further purified by applying to a size exclusion column (HW50, TOSO HAAS, 1.4 x 45 cm). Ck $\beta$ -8 fractionated at a position close to a 13.7Kd molecular weight standard (RNase A), corresponding to the dimeric form of the protein.

Following the three-step purification described above, the resultant Ck $\beta$ -8 was judged to be greater than 90% pure as determined from commassie blue staining of an SDS-PAGE gel (Figure 9).

The purified Ck $\beta$ -8 was also tested for endotoxin/LPS contamination. The LPS content was less than 0.1 ng/ml according to LAL assays (BioWhittaker).

#### Example 11

##### Effect of baculovirus-expressed Ck $\beta$ -1 and Ck $\beta$ -8 on M-CSF and SCF-stimulated colony formation of freshly isolated bone marrow cells.

A low density population of mouse bone marrow cells were incubated in a treated tissue culture dish for one hour at 37°C to remove monocytes, macrophages, and other cells that adhere to the plastic surface. The non-adherent population of cells were then plated (10,000 cells/dish) in agar containing growth medium in the presence or absence of the factors shown in Figure 16. Cultures were incubated for 10 days at 37°C (88% N<sub>2</sub>, 5% CO<sub>2</sub>, and 7% O<sub>2</sub>) and colonies were scored under an inverted microscope. Data is expressed as mean number of colonies and was obtained from assays performed in triplicate.

Example 12Effect of Ck $\beta$ -8 and Ck $\beta$ -1 on IL-3 and SCF stimulated proliferation and differentiation of lin-population of bone marrow cells.

A population of mouse bone marrow cells enriched in primitive hematopoietic progenitors was obtained using a negative selection procedure, where the committed cells of most of the lineages were removed using a panel of monoclonal antibodies (anti cd11b, CD4, CD8, CD45R, and Gr-1 antigens) and magnetic beads. The resulting population of cells (Lin<sup>+</sup> cells) were plated ( $5 \times 10^4$  cells/ml) in the presence or absence of the indicated chemokine (50 ng/ml) in a growth medium supplemented with IL-3 (5 ng/ml) plus SCF (100 ng/ml). After seven days of incubation at 37°C in a humidified incubator (5% CO<sub>2</sub>, 7% O<sub>2</sub>, and 88% N<sub>2</sub> environment), cells were harvested and assayed for the HPP-CFC, and immature progenitors. In addition, cells were analyzed for the expression of certain differentiation antigens by FACScan. Colony data are expressed as mean number of colonies +/- SD) and were obtained from assays performed in six dishes for each population of cells (Figure 17).

Example 13Ck $\beta$ -8 inhibits colony formation in response to IL-3, M-CSF, and GM-CSF.

Mouse bone marrow cells were flushed from both the femur and tibia, separated on a ficol density gradient and monocytes removed by plastic adherence. The resulting population of cells were incubated overnight in an MEM-based medium supplemented with IL-3 (5 ng/ml), GM-CSF (5 ng/ml), M-CSF (10 ng/ml) and G-CSF (10 ng/ml). These cells were plated at 1,000 cells/dish in agar-based colony formation assays in the presence of IL-3 (5 ng/ml), GM-CSF (5 ng/ml) or M-CSF (5 ng/ml) with or without Ck $\beta$ -8 at 50 ng/ml. The data is presented as colony formation as a percentage of the number

of colonies formed with the specific factor alone. Two experiments are shown with the data depicted as the average of duplicate dishes with error bars indicating the standard deviation for each experiment (Figure 19).

#### Example 14

##### Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer having contains a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for

ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: LI, ET AL.

(ii) TITLE OF INVENTION: Human Chemokine Beta-8,  
Chemokine Beta-1 and  
Macrophage Inflammatory  
Protein-4

(iii) NUMBER OF SEQUENCES: 18

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN

(B) STREET: 6 BECKER FARM ROAD

(C) CITY: ROSELAND

(D) STATE: NEW JERSEY

(E) COUNTRY: USA

(F) ZIP: 07068

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 INCH DISKETTE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WORD PERFECT 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/173,209

(B) FILING DATE: 22 DEC 93

(viii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/208,339

(B) FILING DATE: 08 MAR 94

(ix) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US94/07256

(B) FILING DATE: 28 JUNE 1994

(ix) ATTORNEY/AGENT INFORMATION:

(A) NAME: FERRARO, GREGORY D.

(B) REGISTRATION NUMBER: 36,134

(C) REFERENCE/DOCKET NUMBER: 325800-289

(x) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201-994-1700

(B) TELEFAX: 201-994-1744

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 363 BASE PAIRS  
 (B) TYPE: NUCLEIC ACID  
 (C) STRANDEDNESS: SINGLE  
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

ATGAAGGTCT CCGTGGCTGC CCTCTCCTGC CTCATGCTTG TTACTGCCCT TGGATCCCAG 60
GCCCCGGGTCA CAAAAGATGC AGAGACAGAG TTCATGATGT CAAAGCTTCC ATTGGAAAAT 120
CCAGTACTTC TGGACAGATT CCATGCTACT AGTGCTGACT GCTGCATCTC CTACACCCCA 180
CGAAGCATCC CGTGTTCCT CCTGGAGAGT TACTTTGAAA CGAACAGCGA GTGCTCCAAG 240
CCGGGTGTCA TCTTCCTCAC CAAGAAGGGG CGACGTTTCT GTGCCAACCC CAGTGATAAG 300
CAAGTTCAGG TTTGCATGAG AATGCTGAAG CTGGACACAC GGATCAAGAC CAGGAAGAAT 360
TGA

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 120 AMINO ACIDS  
 (B) TYPE: AMINO ACID  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Lys Val Ser Val Ala Ala Leu Ser Cys Leu Met Lys Val Thr
-20 -15 -10
Ala Leu Gly Ser Gln Ala Arg Val Thr Lys Asp Ala Glu Thr Glu
-5 1 5
Phe Met Met Ser Lys Leu Pro Leu Glu Asn Pro Val Leu Leu Asp
10 15 20
Arg Phe His Ala Thr Ser Ala Asp Cys Cys Ile Ser Tyr Thr Pro
25 30 35
Arg Ser Ile Pro Cys Ser Leu Leu Glu Ser Tyr Phe Glu Thr Asn
40 45 50
Ser Glu Cys Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Lys Gly
55 60 65
Arg Arg Phe Cys Ala Asn Pro Ser Asp Lys Gln Val Gln Val Cys
70 75 80
Met Arg Met Leu Lys Leu Asp Thr Arg Ile Lys Thr Arg Lys Asn
85 90 95

```

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 282 BASE PAIRS

(B) TYPE: NUCLEIC ACID  
 (C) STRANDEDNESS: SINGLE  
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

ATGAAGATCT CCGTGGCTGC AATTCCCTTC TTCCTCCTCA TCACCATCGC CCTAGGGACC 60
AAGACTGAAT CCTCCTCAG GGGACCTTAC CACCCCTCAG AGTGCTGCTT CACCTACACT 120
ACCTACAAGA TCCCGCGTCA GCGGATTATG GATTACTATG AGACCAACAG CCAGTGCTCC 180
AAGCCCGGAA TTGTCTTCAT CACCAAAAGG GGCCATTCCG TCTGTACCAA CCCAGTGAC 240
AAGTGGGTCC AGGACTATAT CAAGGACATG AAGGAGAACT GA 282
  
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS  
 (A) LENGTH: 93 AMINO ACIDS  
 (B) TYPE: AMINO ACID  
 (C) STRANDEDNESS:  
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Lys Ile Ser Val Ala Ala Ile Pro Phe Phe Leu Leu Ile Thr
      -15                      -10                      -5
Ile Ala Leu Gly Thr Lys Thr Glu Ser Ser Ser Arg Gly Pro Tyr
      1                      5                      10
His Pro Ser Glu Cys Cys Phe Thr Tyr Thr Thr Tyr Lys Ile Pro
      15                      20                      25
Arg Gln Arg Ile Met Asp Tyr Tyr Glu Thr Asn Ser Gln Cys Ser
      30                      35                      40
Lys Pro Gly Ile Val Phe Ile Thr Lys Arg Gly His Ser Val Cys
      45                      50                      55
Thr Asn Pro Ser Asp Lys Trp Val Gln Asp Tyr Ile Lys Asp Met
      60                      65                      70
Lys Glu Asn
  
```

(3) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS  
 (A) LENGTH: 270 BASE PAIRS  
 (B) TYPE: NUCLEIC ACID  
 (C) STRANDEDNESS: SINGLE  
 (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

ATGAAGGGCC TTGCAGCTGC CTCCTTGTG CTCGTCTGCA CCATGGCCCT CTGCTCCTGT 60
  
```



```

GCACAAGTTG GTACCAACAA AGAGCTCTGC TGCCTCGTCT ATACCTCCTG GCAGATTCCA 120
CAAAAGTTCA TAGTTGACTA TTCTGAAACC AGCCCCCAGT GCCCAAGCC AGGTGTCATC 180
CTCCTAACCA AGAGAGGCCG GCAGATCTGT GCTGACCCCA ATAAGAAGTG GGTCCAGAAA 240
TACATCAGCG ACCTGAAGCT GAATGCCTGA 270

```

## (4) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 89 AMINO ACIDS
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS:
- (D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: PROTEIN

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Lys Gly Leu Ala Ala Ala Leu Leu Val Leu Val Cys Thr Met
-20          -15          -10
Ala Leu Cys Ser Cys Ala Gln Val Gly Thr Asn Lys Glu Leu Cys
-5          1          5          10
Cys Leu Val Tyr Thr Ser Trp Gln Ile Pro Gln Lys Phe Ile Val
15          20          25
Asp Tyr Ser Glu Thr Ser Pro Gln Cys Pro Lys Pro Gly Val Ile
30          35          40
Leu Leu Thr Lys Arg Gly Arg Gln Ile Cys Ala Asp Pro Asn Lys
45          50          55
Lys Trp Val Gln Lys Tyr Ile Ser Asp Leu Lys Leu Asn Ala
60          65

```

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 26 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

## (ii) MOLECULE TYPE: Oligonucleotide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCAGGATCCG TCACAAAAGA TGCAGA

26

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 26 BASE PAIRS
- (B) TYPE: NUCLEIC ACID
- (C) STRANDEDNESS: SINGLE
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCTCTAGAG TAAAACGACG GCCAGT

26

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 27 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCAGGATCCT GTGCACAAGT TGGTACC

27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 26 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCTCTAGAG TAAAACGACG GCCAGT

26

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 30 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCCGCGGAT CTCCTCACG GGGACCTTAC

30

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 32 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCCTGCTCTA GATCAAAGCA GGGAAGCTCC AG

32

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 27 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAAAGCTTA TGAAGGTCTC CGTGGCT

27

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 59 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAA TTCTTCCTGG TCTTGATCC 59

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 30 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGAAAGCTTA TGAAGGGCCT TGCAGCTGCC

30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 57 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGCTCTAGAT CAABCGTAGT CTGGGACGTC GTATGGGTAG GCATTCAGCT TCAGGTC  
57

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 28 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGAAAGCTTA TGAAGATTCC GTGGCTGC

28

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 58 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAG TTCTCCTTCA TGTCCTTG 58

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide encoding the polypeptide comprising amino acids -21 to amino acid 99 of SEQ ID No. 2;
  - (b) a polynucleotide encoding the polypeptide comprising amino acids 1 to amino acid 99 of SEQ ID No. 2;
  - (c) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a) or (b); and
  - (d) a polynucleotide fragment of the polynucleotide of (a) or (b).
2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.
4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.
5. The polynucleotide of Claim 2 which encodes the polypeptide comprising amino acid -21 to 99 of SEQ ID No. 2.
6. The polynucleotide of Claim 2 which encodes the polypeptide comprising amino acid 1 to 99 of SEQ ID No. 2.
7. An isolated polynucleotide comprising a member selected from the group consisting of:
  - (a) a polynucleotide which encodes a polypeptide having the amino acid sequence expressed by the DNA contained in ATCC Deposit No. 75676;
  - (b) a polynucleotide capable of hybridizing to and which is at least 70% identical to the polynucleotide of (a);

(c) a polynucleotide fragment of the polynucleotide of (a) or (b).

8. The polynucleotide of claim 1 comprising the sequence as set forth in SEQ ID No. 1 from nucleotide 1 to nucleotide 363.

9. A vector containing the DNA of Claim 2.

10. A host cell genetically engineered with the vector of Claim 9.

11. A process for producing a polypeptide comprising: expressing from the host cell of Claim 10 the polypeptide encoded by said DNA.

12. A process for producing cells capable of expressing a polypeptide comprising genetically engineering cells with the vector of Claim 9.

13. A polypeptide selected from the group consisting of (i) a polypeptide having the deduced amino acid sequence of SEQ ID No. 2 and fragments, analogs and derivatives thereof; and (ii) a polypeptide encoded by the cDNA of ATCC Deposit No. 75676 and fragments, analogs and derivatives of said polypeptide.

14. The polypeptide of Claim 13 wherein the polypeptide has the deduced amino acid sequence of SEQ ID No. 2.

15. An agonist for the polypeptide of claim 13.

16. An antagonist against the polypeptide of claim 13.

17. A method for the treatment of a patient having need of Ck $\beta$ -8 comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 13.

18. The method of Claim 17 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
19. A method for the treatment of a patient having need to inhibit a Ck $\beta$ -8 polypeptide comprising: administering to the patient a therapeutically effective amount of the antagonist of Claim 16.
20. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 13 comprising:  
determining a mutation in the nucleic acid sequence encoding said polypeptide.
21. A diagnostic process comprising:  
analyzing for the presence of the polypeptide of claim 13 in a sample derived from a host.
22. A process for identifying antagonists and agonists to the polypeptide of claim 13 comprising:  
combining cells, a compound to be screened and said polypeptide wherein the cells are separated from said polypeptide by a porous filter; and  
determining the extent of migration of the cells to determine if the compound is an effective antagonist or agonist.

## FIG. 1

ATGAAGGTCCTCCGTGGCTGCCCTCTCCTGCCCTCATGCTTGTTACTGCCCTTGGATCCCAG 60  
M K V S V A A L S C L M L V T A L G S Q

GCCCGGTCACAAAGATGCAGACAGAGTCCATGATGTCAAAGCTTCCATTGGAAAAT 120  
A R V T K D A E T E F M M S K L P L E N

CCAGTACTTCTGGACAGATTCCATGCTACTAGTGTGCTGACTGCTGCATCTCCTACACCCCA 180  
P V L L D R F H A T S A D C C I S Y T T

CGAAGCATCCCGTGTTCACTCCTGGAGAGTTACTTTGAACGACGAGTGCTCCAAG 240  
R S I P C S L L E S Y F E T N S E C S K

CCGGGTGTCATCTTCCTCACCAAGAAGGGGCGACGTTTCTGTGCCAACCCAGTGATAAG 300  
P G V I F L T K K G R R F C A N P S D K

CAAGTTCAGGTTTGCCATGAGAATGCTGAAGCTGGACACACGGATCAAGACCAGGAAGAAT 360  
Q V Q V C M R M L L K L D T R I K T R K N

TGA 363

\*



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## FIG. 2

ATGAAGATCTCCGTGGCTGCAATTCCCTTCTTCCTCCTCATCACCATCGCCCCCTAGGGACC  
M K I S V A A I P F F L L I T I A L G T

AAGACTGAATCCTCCTCACGGGGACCTTACCACCCCTCAGAGTGTCTTCACTACACT  
K T E S S S R G P Y H P S E C C F T Y T

ACCTACAAGATCCCGGTCAGCGGATTATGGATTACTATGAGACCAACAGCCAGTGTCTCC  
T Y K I P R Q R I M D Y Y E T N S Q C S

AAGCCCGGAATTGTCTTCATCACCAAAAGGGGCCATTCCGTCTGTACCAACCCCAAGTGAC  
K P G I V F I T K R G H S V C T N P S D

AAGTGGTCCAGGACTATATCAAGGACATGAAGGAGAACTGA  
K W V Q D Y I K D M K E N \*

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## FIG. 3

1 ATGAAAGGCGCTTGCAGCTGCCCTCCTTGTCCCTCGTCTGCACCATGGCCCTCTGCTCCTGT 60  
M K G L A A A L L V L V C T M A L C S C .

61 GCACAAGTTGTGACCAACAAGAGCTCTGCTGCCCTCGTCTATACCTCCTGGCAGATTCCA 120  
A Q V G T N K E L C C L V Y T S W Q I P

121 CAAAGTTCATAGTTGACTATTCTGAAACCAGCCCCCAGTCCCCCAAGCCAGTGGTCATC 180  
Q K F I V D Y S E T S P Q C P K P G V I

181 CTCCCTAACCAAGAGAGGCGCGCAGATCTGTGCTGACCCCCCAATAAGAAGTGGGTCCAGAAA 240  
L L T K R G R Q I C A D P N K K W V Q K

241 TACATCAGCGACCTGAAGCTGAATGCCCTGA 270  
Y I S D L K L N A \*

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## FIG. 4

CK $\beta$ -8	MKVSVAALSCLMLVLTALGSQARVTKDAETEFMMSKLPLENPVLLDRFHAT	50
	I.II.III.II: . III.I . . . . I:	
MIP-1 $\alpha$	MQVSTAALAVLLCTMALCNQFSASLAAD.....T	29
CK $\beta$ -8	SADCCISYTPRSIPCSLLESYFETNSECSKPGVIFLTKKGRRFCANPSDK	100
	...II:III.I II .:..IIII.I:IIIIIIII:..II:II:..	
MIP-1 $\alpha$	PTACCFYSYTSRQIPQNFADYFETSSQCSKPGVIFLTKRSRQVCADPSEE	79
CK $\beta$ -8	QVQVCMRMLKLDTRIKTRKN	120
	II :.: I.I..	
MIP-1 $\alpha$	WVQKYVSDLELSA	92

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## FIG. 5

1 MKGLAAALVLVCTMALC...SCAQVGTNKELCCLVYTSWQIPQKFIVD 46

1 MQVSTAALAVLLCTMALCNQVLSAPLAADPTTACCFSTSRQIPQNFAD 50

47 YSETSPQCPKPGVILLTKRGRQICADPNKKWVQKYISDLKLNA 89

51 YFETSSQCSKPSVIFLTKRGRQVCADPSEEWVQKYVSDLELSA 93

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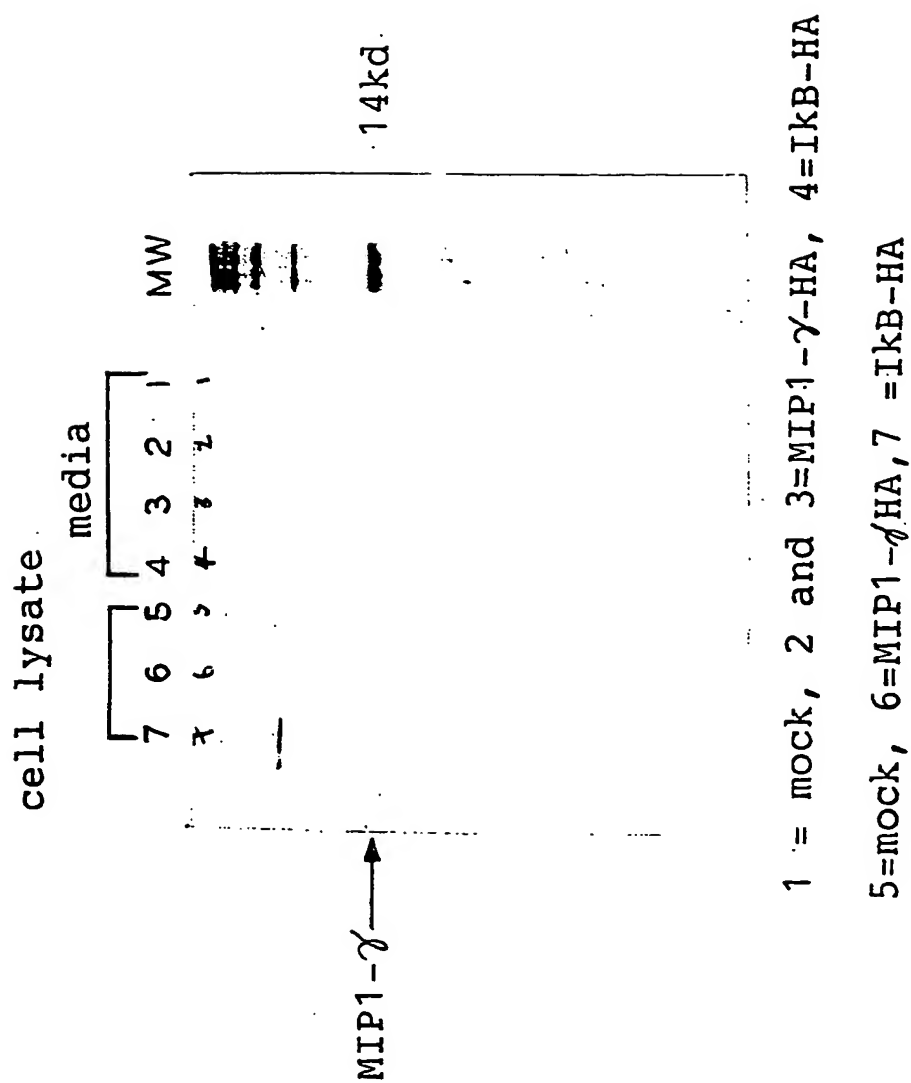
**6/22**

6. G. 1. E.

[illegible]

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## FIG. 7

Expression of HA-Tagged MIP1- $\gamma$  in Cos Cells

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Expression and purification of CK $\beta$ -1 in a baculovirus expression system.

Purification of CK $\beta$ -1

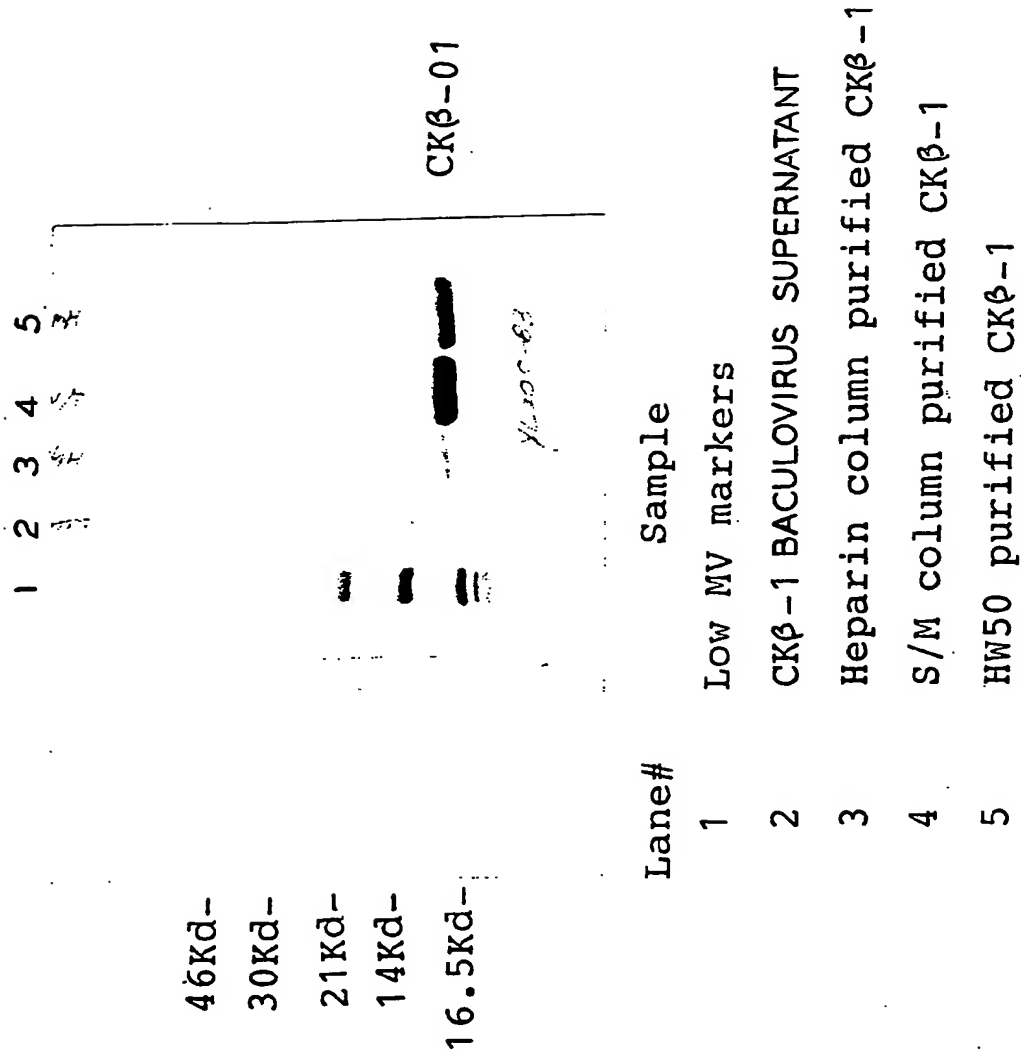
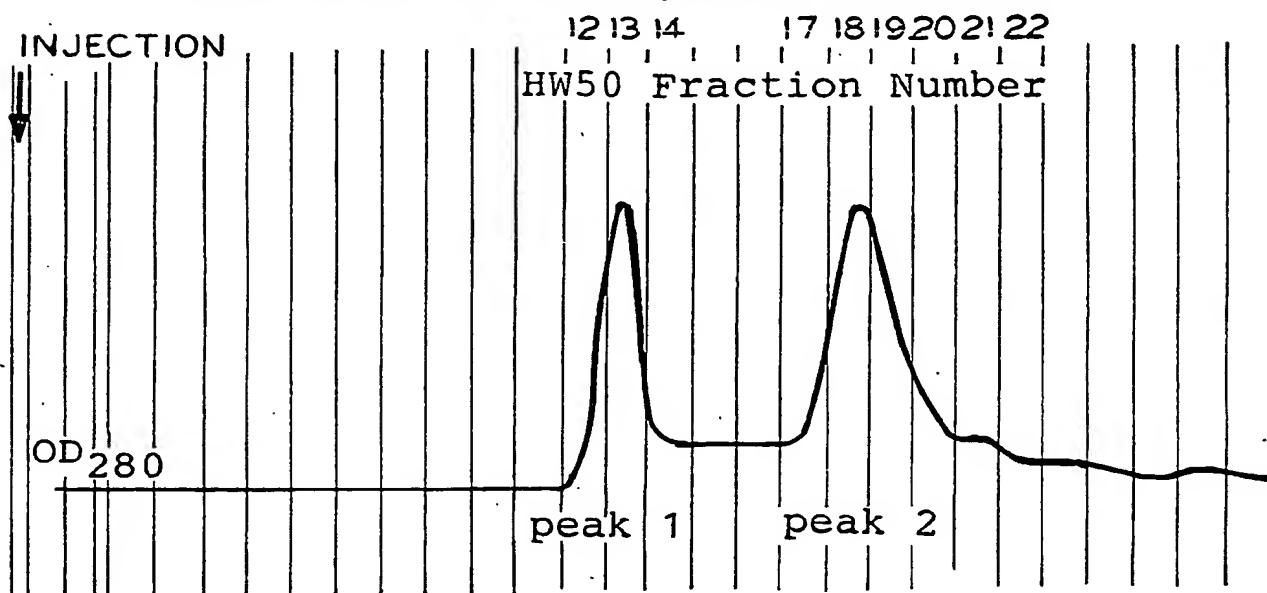


FIG. 8

Purification of CK $\beta$ -8 (HG301-4B) on size  
exclusion chromatography (Step 3).



15% SDS-PAGE

46Kd—  
30Kd—  
21Kd—  
14Kd—  
6.5Kd—

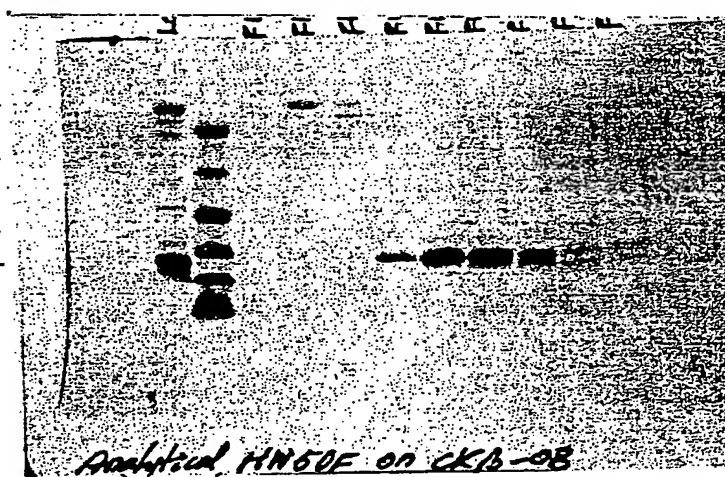


FIG. 9

YL200-41

peak 1 peak 2

Lane# Sample

1	HW50 load	
2	Low MW markers	12
3	HW50 fraction	13
4		14
5		15
6		16
7		17
8		18
9		19
10		21
11		22

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FIG. 10A

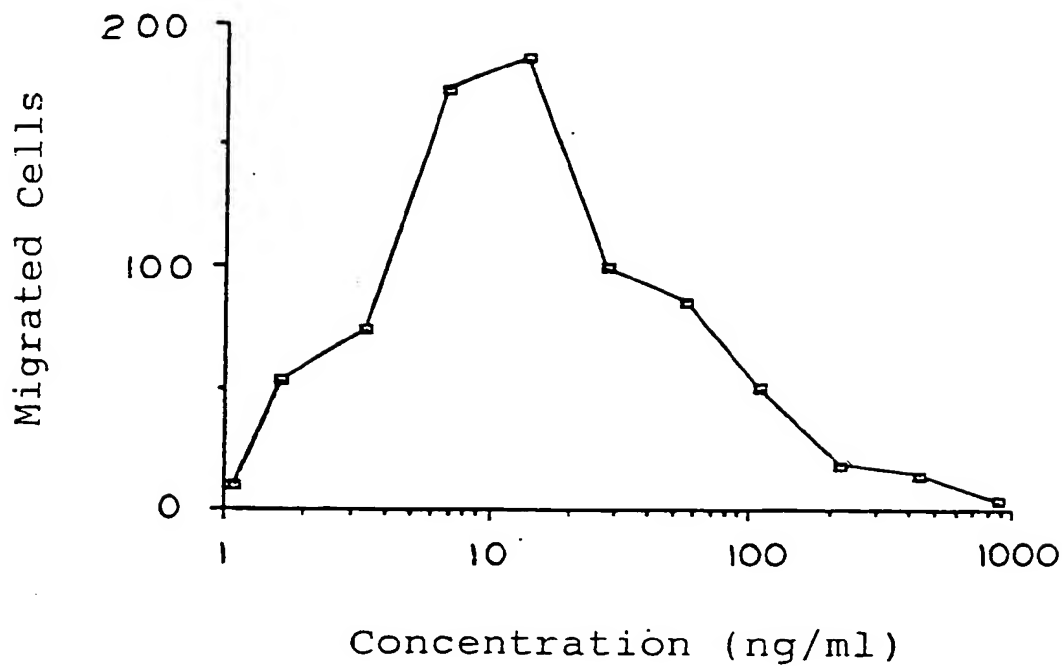
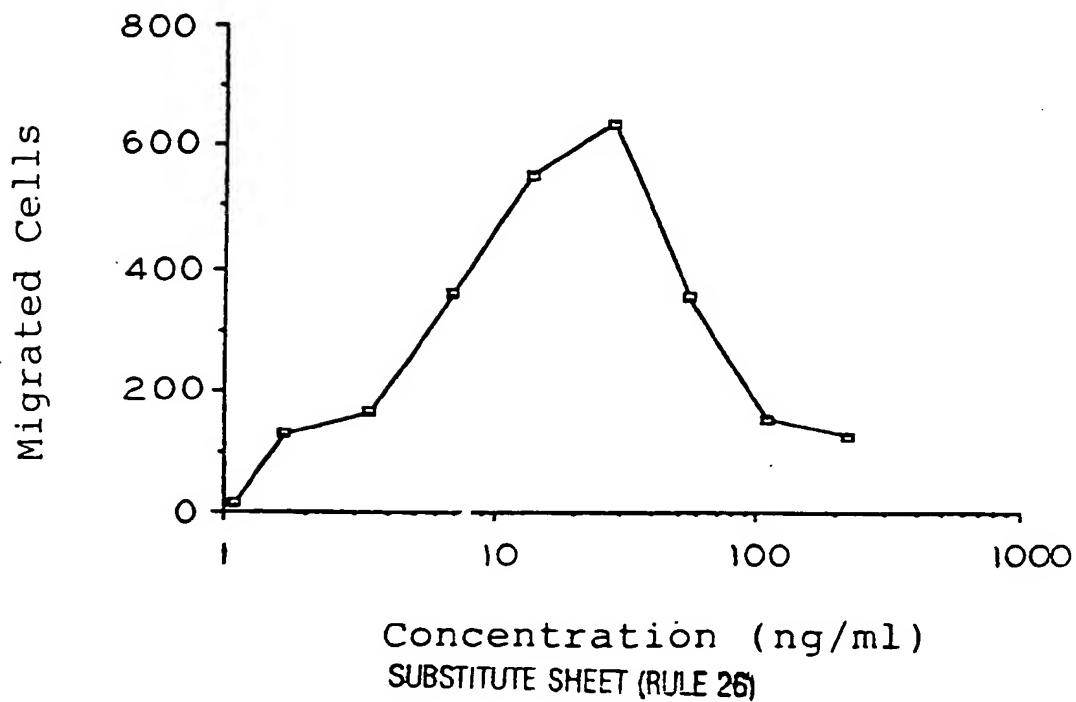
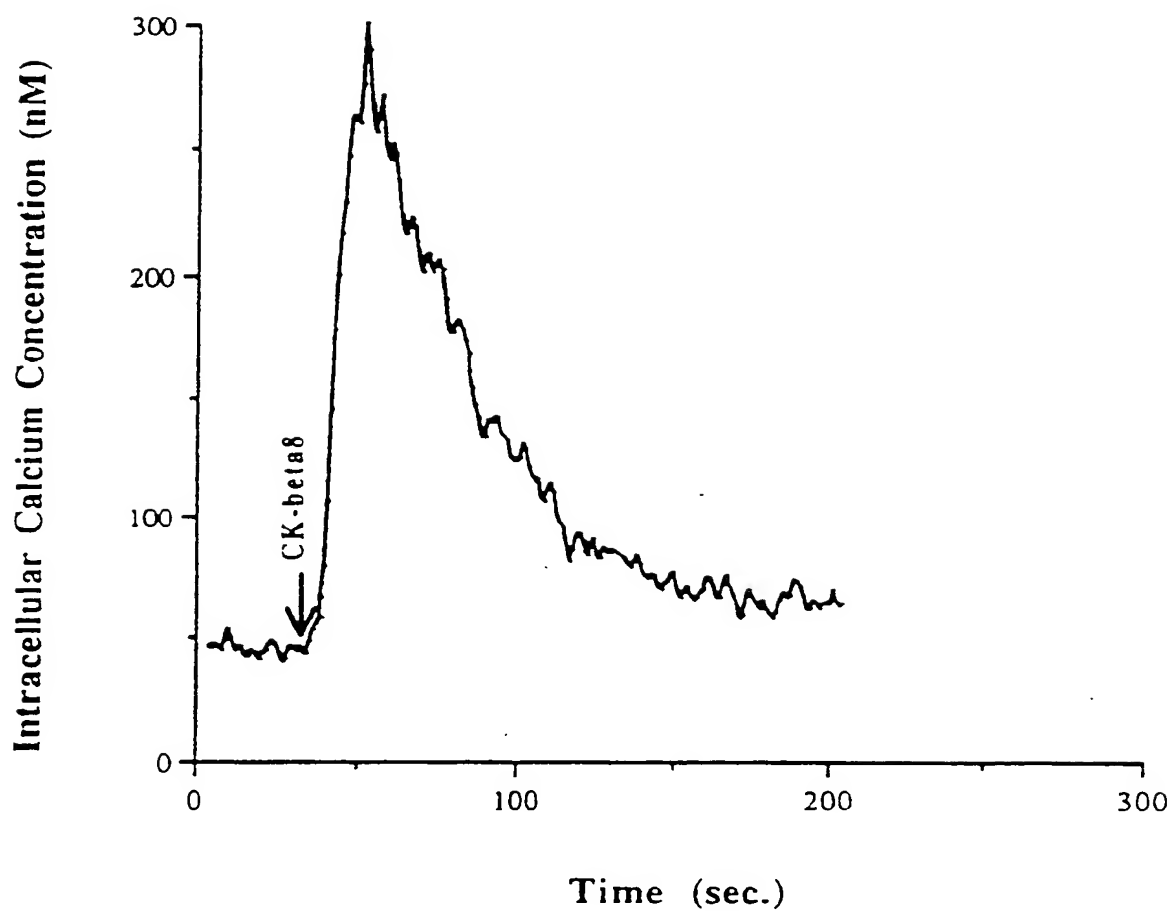


FIG. 10B



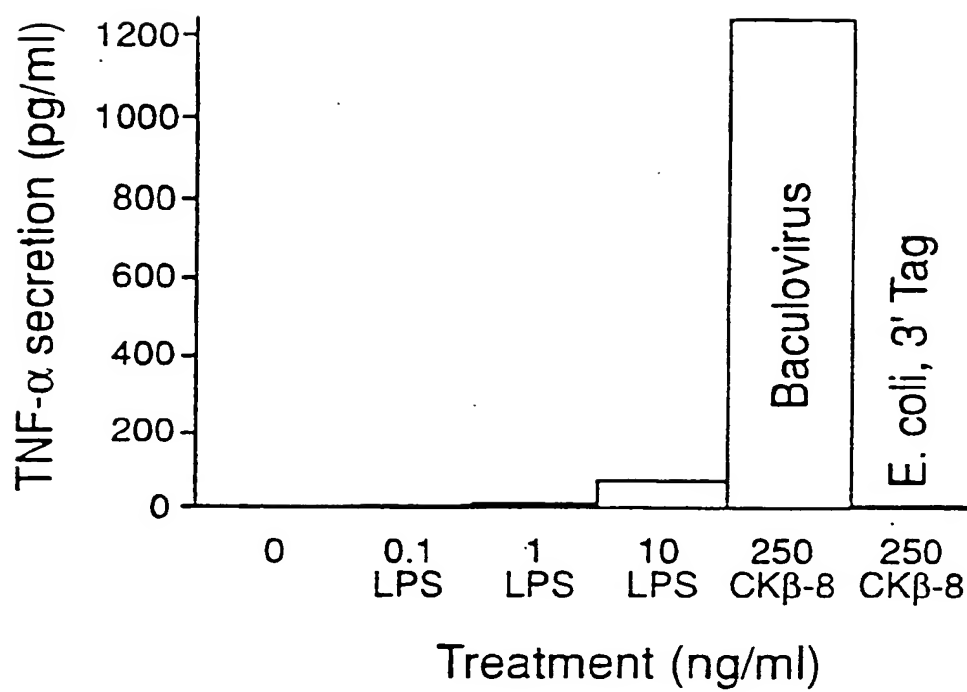
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FIG. 11



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FIG. 12



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FIG. 13A

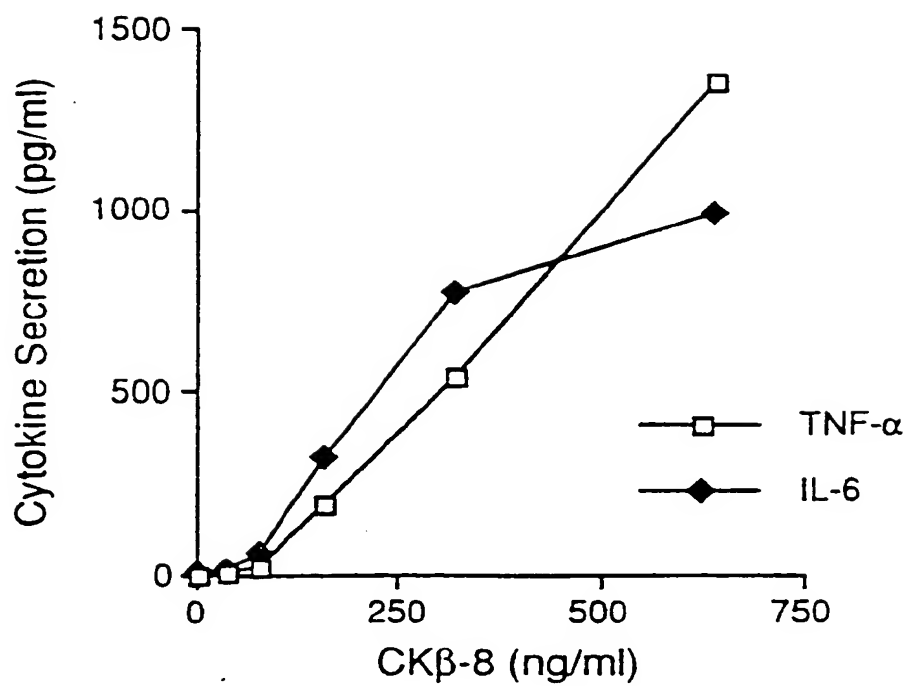
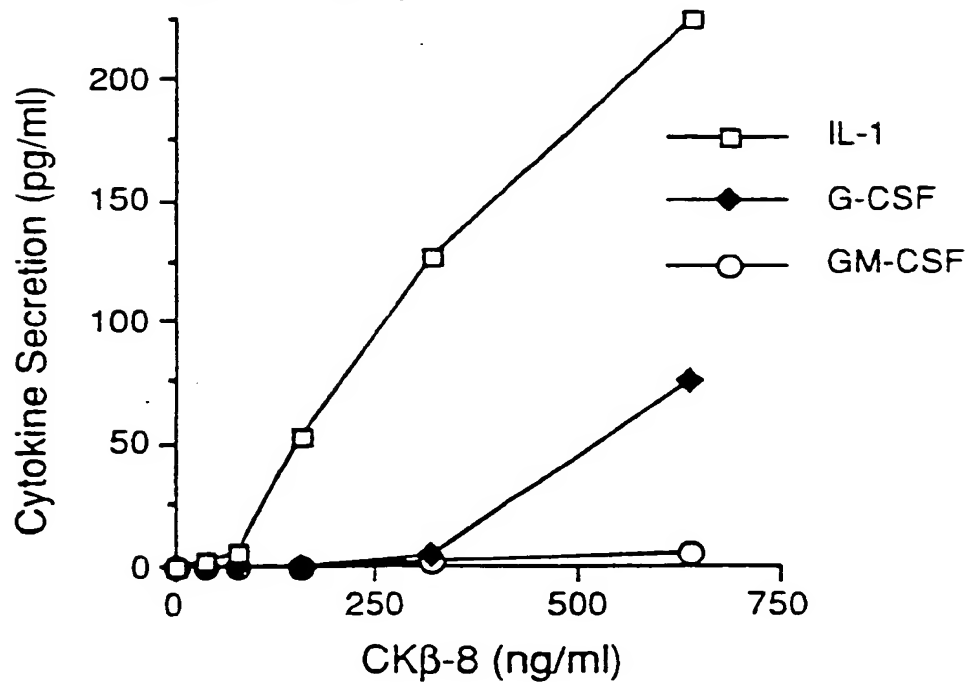


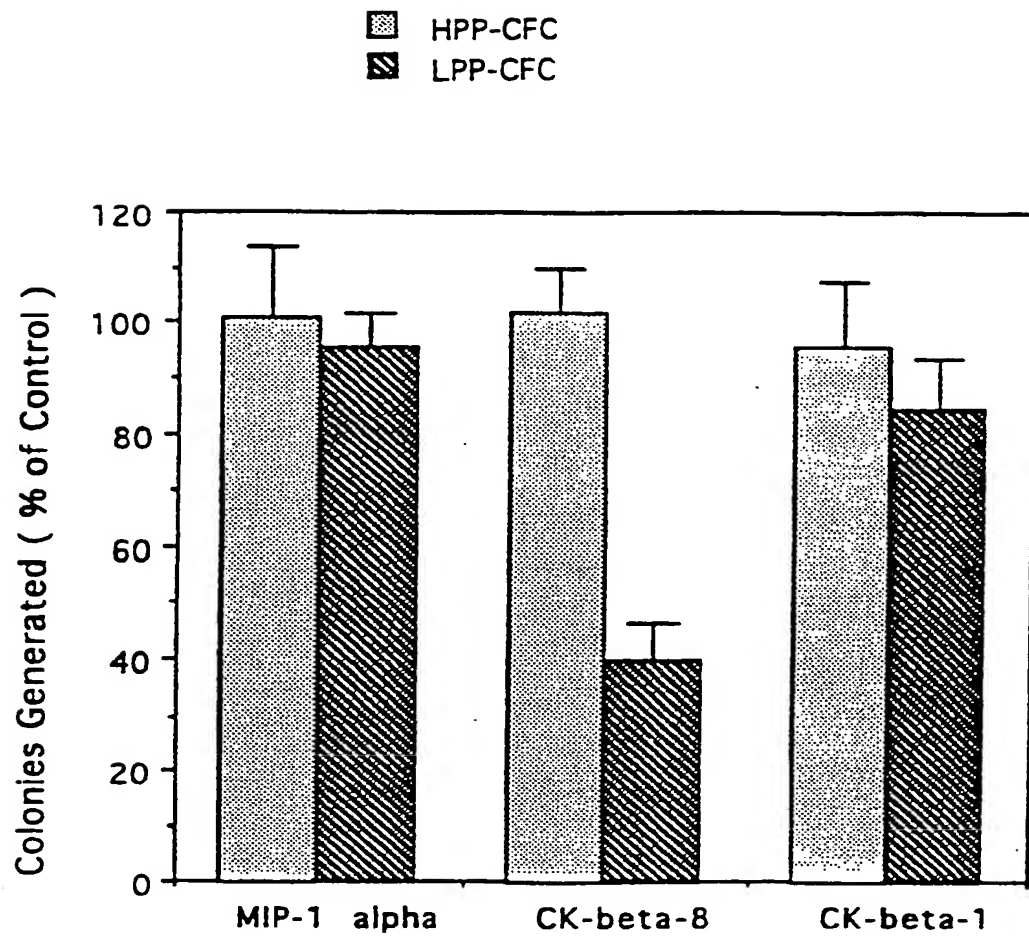
FIG. 13B



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FIG. 14



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FIG. 15A

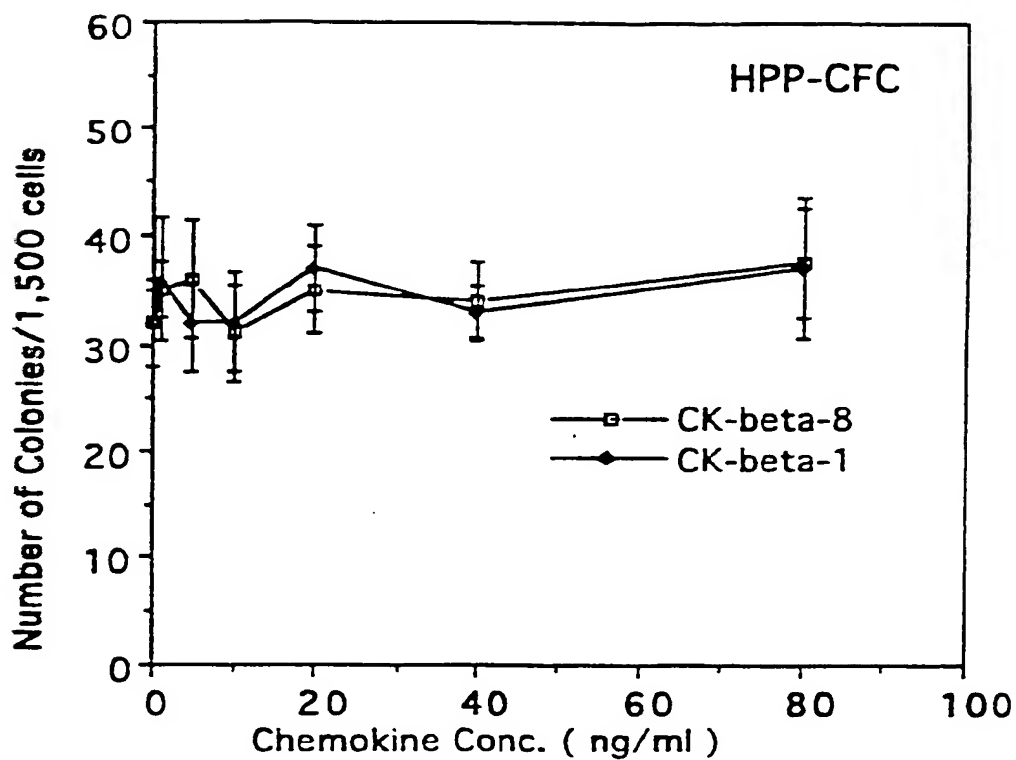
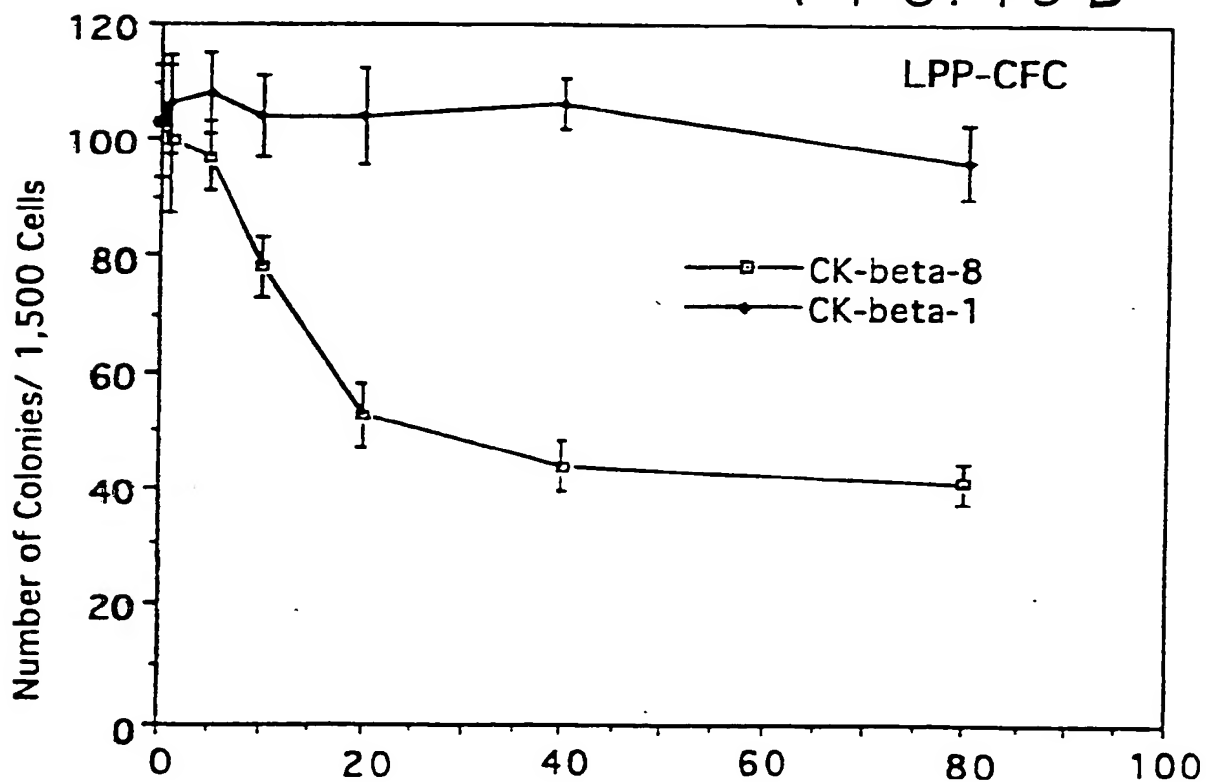


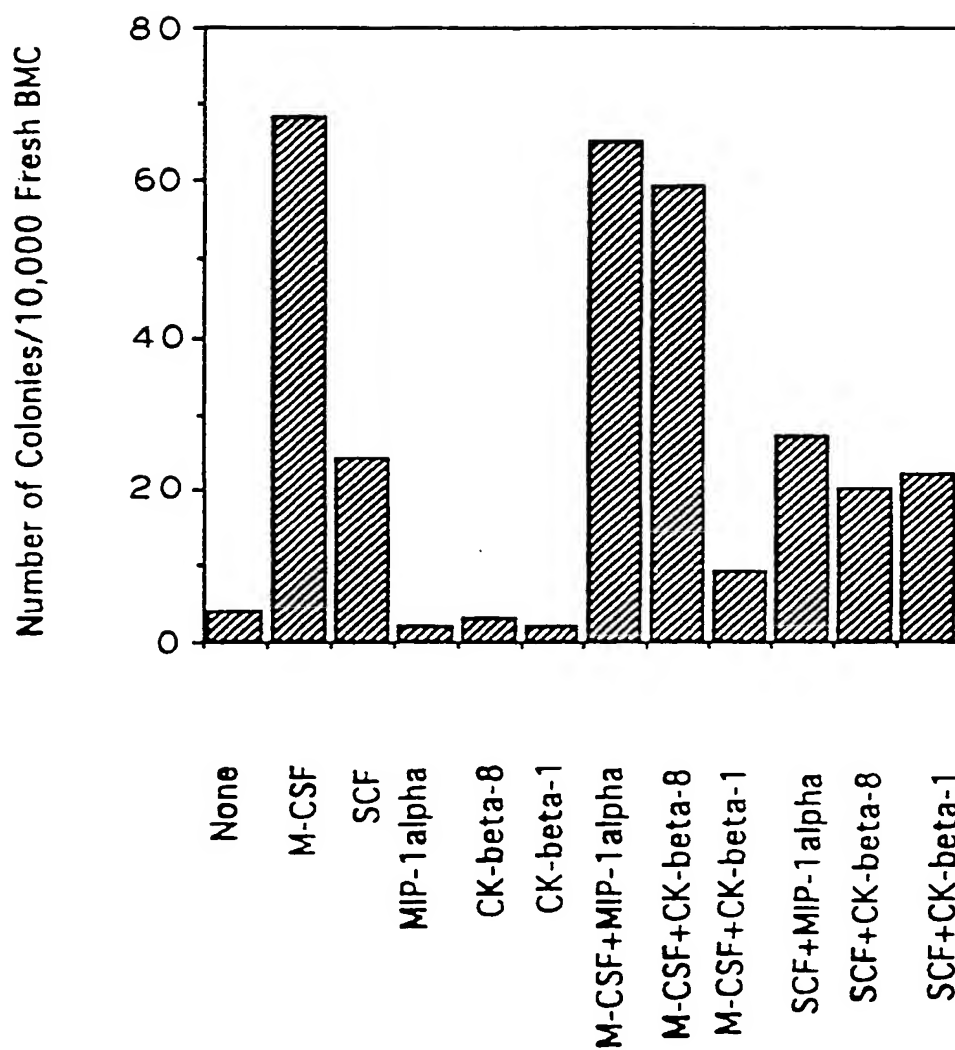
FIG. 15B



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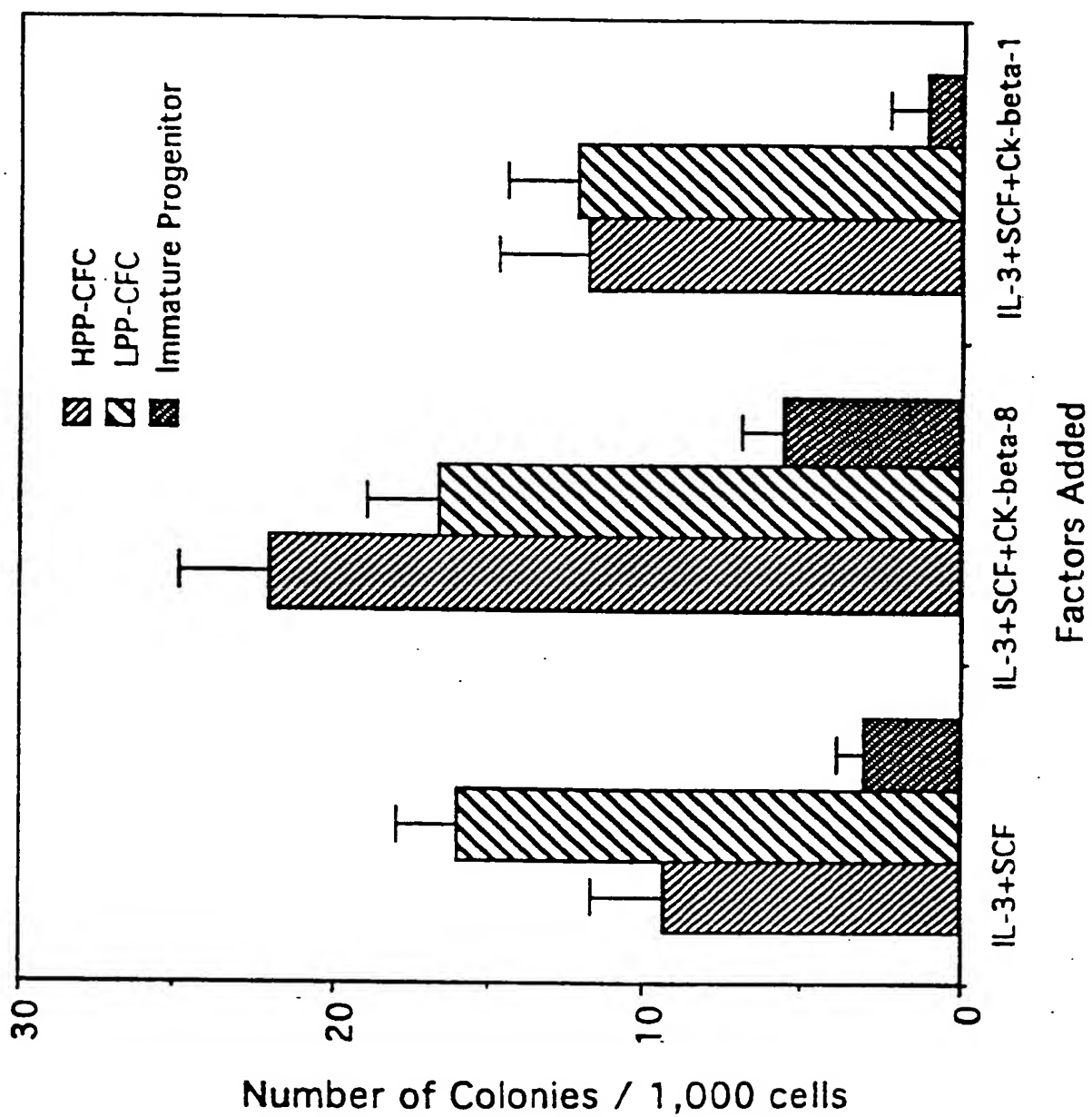
FIG. 16



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FIG. 17



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FIG. 18A

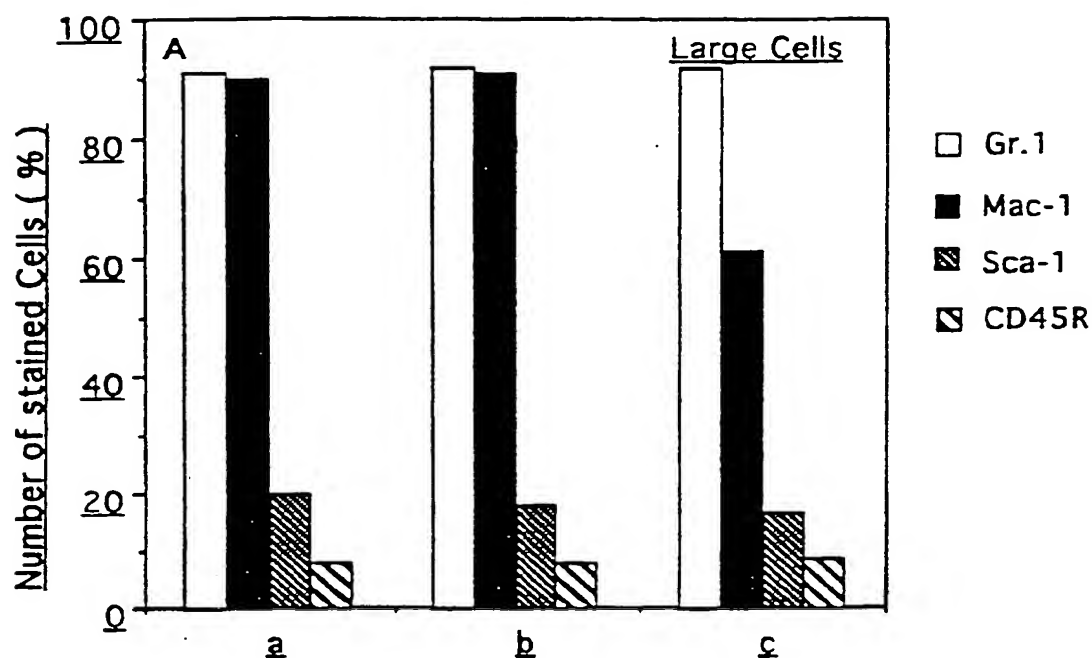
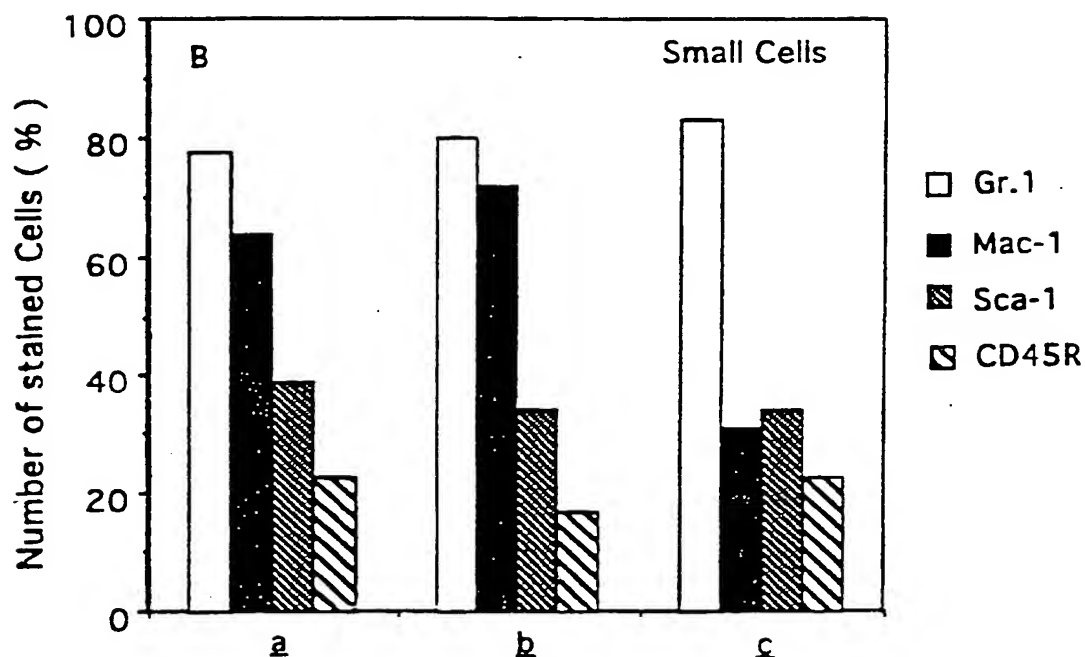


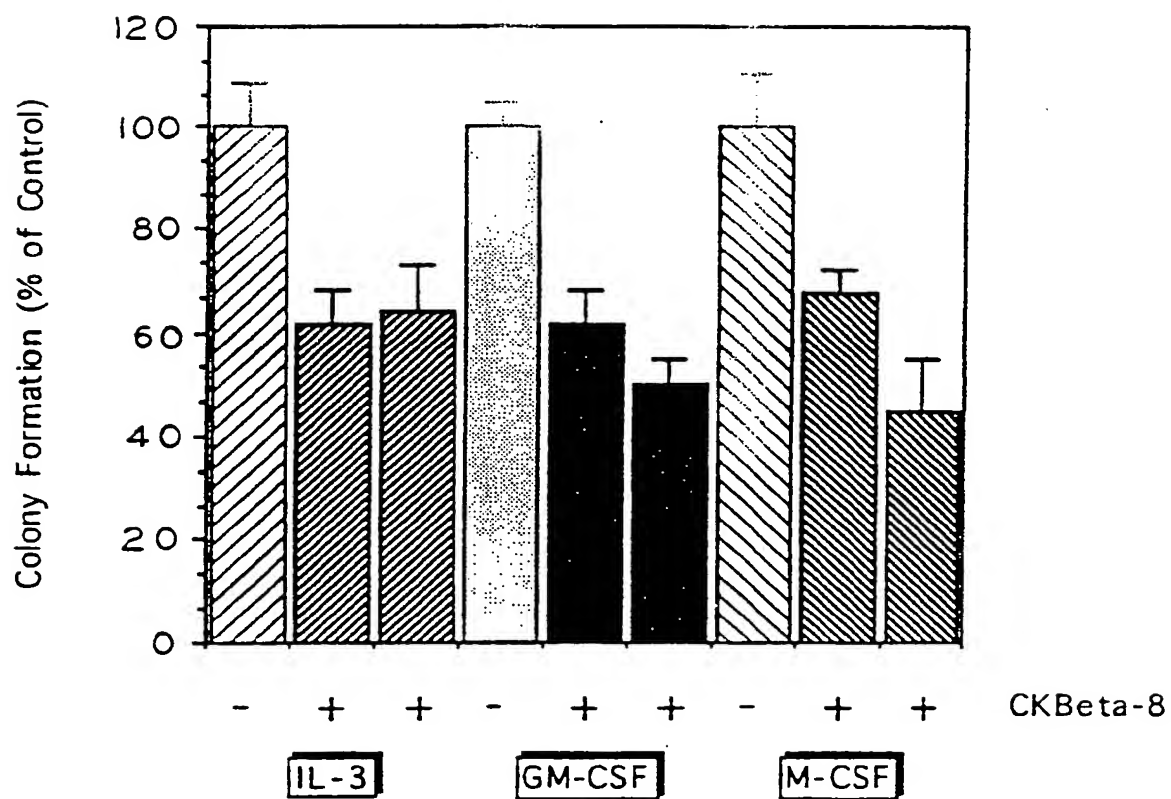
FIG. 18B



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1992

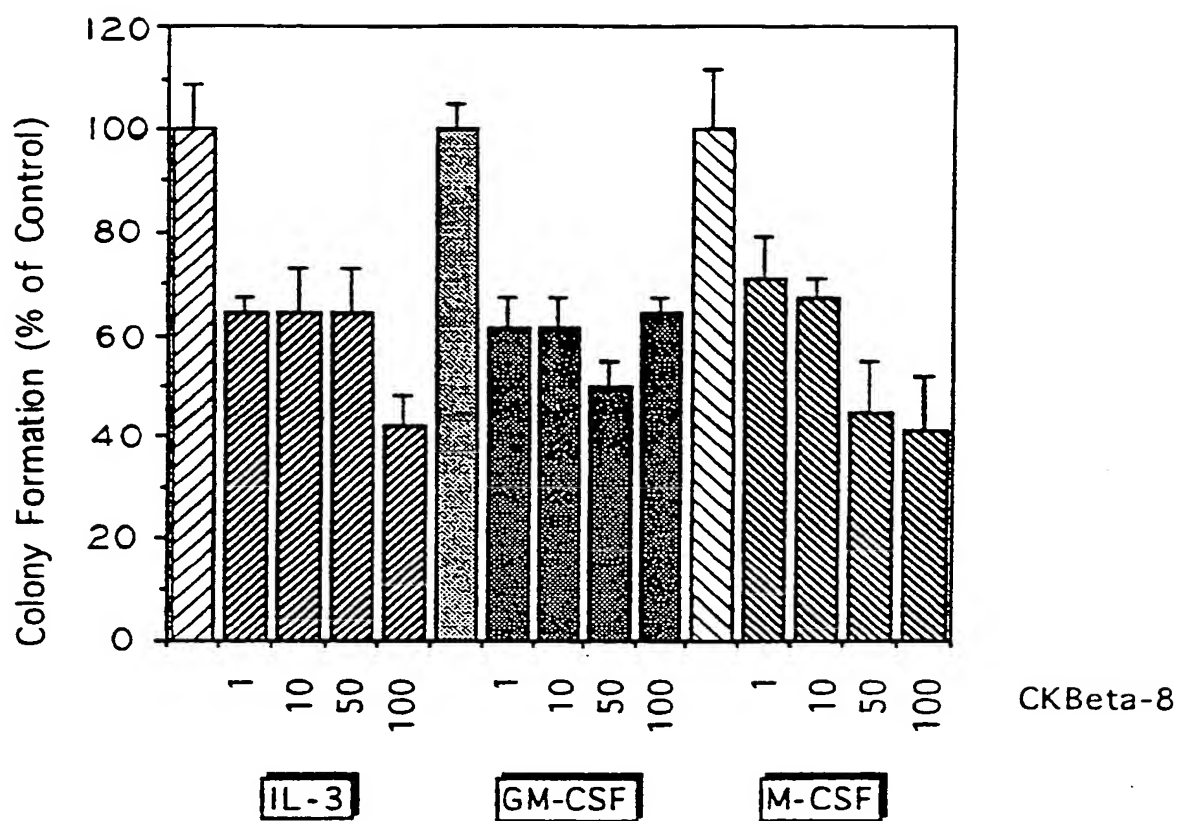
FIG. 19



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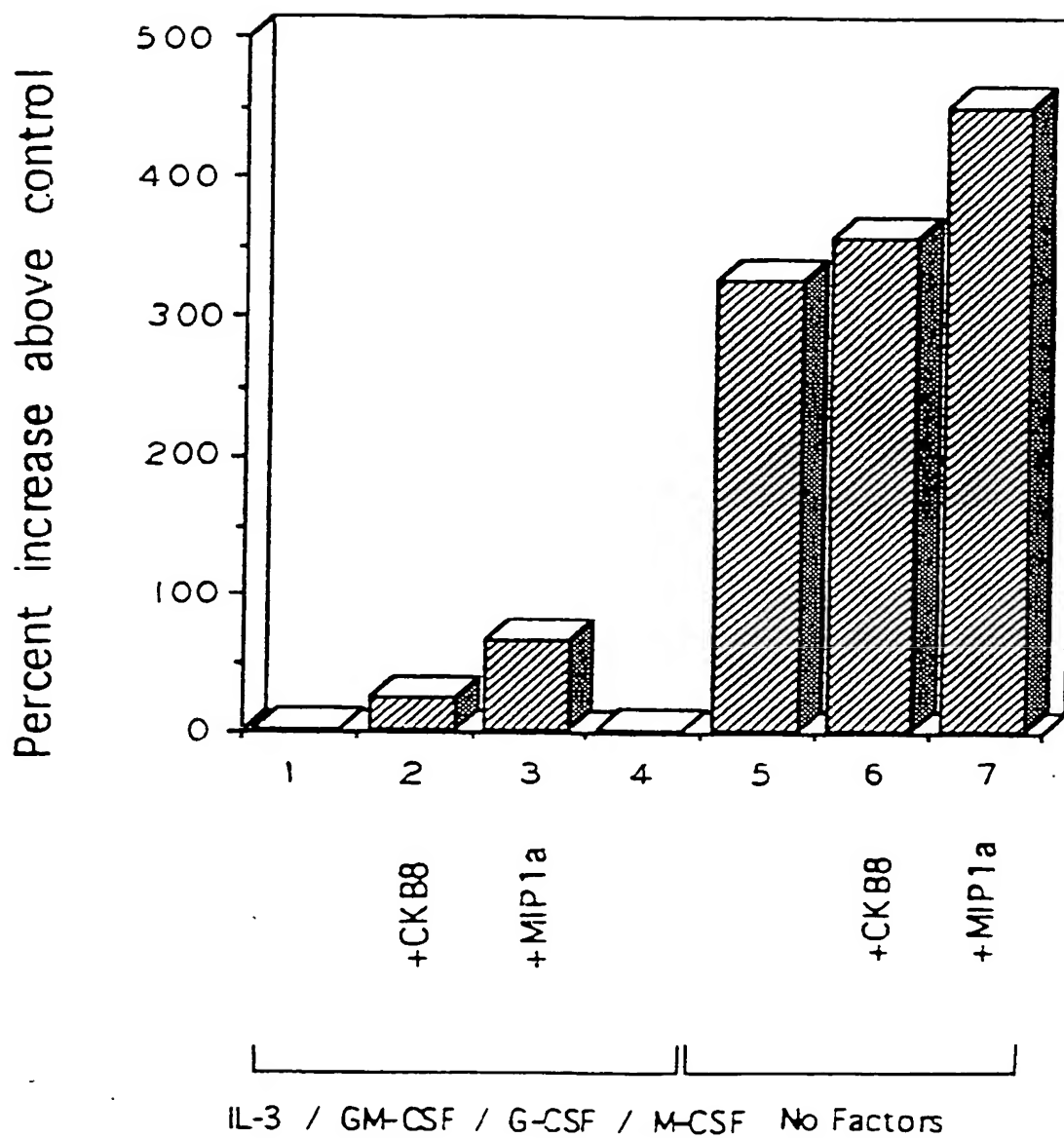
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FIG. 20



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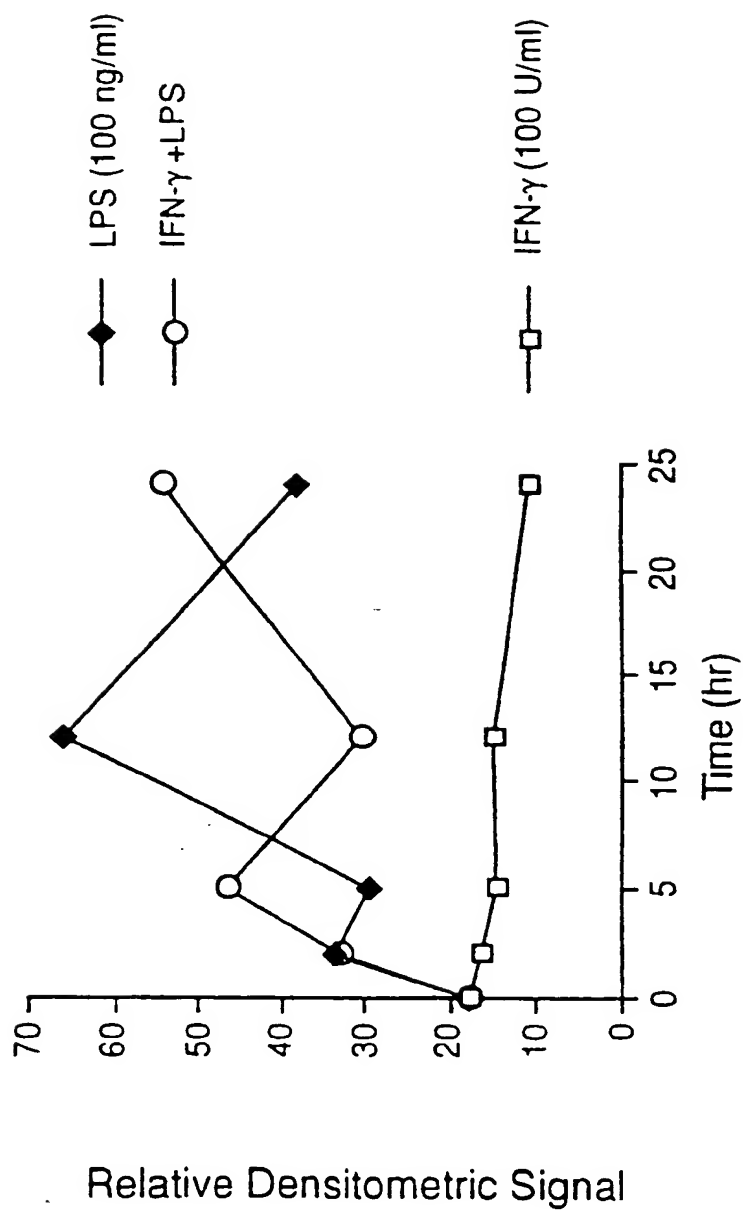
FIG. 21



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FIG. 22



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/09058

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 16/24; C07K 14/52; C12N 1/20, 15/09, 15/19, 15/63

US CL : 536/23.1; 530/351; 435/69.1, 252.3, 320.1; 514/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/351; 435/69.1, 252.3, 320.1; 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS, SCISEARCH

search terms: chemokines, beta-8, beta-1, macrophage inflammatory protein-4, production or isolation, treatment, therapy.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FEBS LETTERS, Volume 360, issued 1995, Yoshida et al, "Molecular Cloning of a Novel C or Gamma Type Chemokine, SCM-1", pages 155-159, see page 155, column 1, see abstract.	1-14
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 269, Number 41, issued 14 October 1994, Chang et al, "Cloning and Characterization of the Human Neutrophil-activating Peptide (ENA-78) Gene", pages 25277-25282, see page 25277, column 2, lines 10-21.	1-14
A	GENE, Volume 151, issued 1994, Power et al, "Cloning of a Full-length cDNA Encoding the Neutrophil-activating Peptide ENA-78 from Human Platelets", pages 333-334, see page 333, see abstract.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "A" document defining the general state of the art which is not considered to be of particular relevance	* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* "E" earlier document published on or after the international filing date	* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* "&" document member of the same patent family
* "O" document referring to an oral disclosure, use, exhibition or other means	
* "P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 18 SEPTEMBER 1995	Date of mailing of the international search report 19 OCT 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PREMA MERTZ
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/09058

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMISTRY, Volume 33, issued 1994, Proost et al, "Purification , Sequence Analysis, and Biological Characterization of a Second Bovine Monocyte Chemotactic Protein-1 (Bo MCP-1B)", pages 13406-13412, see page 13406, column 1, lines 12-25.	1-14
A	GENE, Volume 151, issued 1994, Johnson II et al, "Cloning of Two Rabbit GRO Homologues and Their Expression in Alveolar Macrophages", pages 337-338, see page 338, column 2, lines 1-9.	1-14, 17

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/09058

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-14 and 17

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)\*



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/09058

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims, 1-14 and 17, drawn to a polynucleotide, a vector, a host cell, a process for producing a polynucleotide, a process for producing cells, a polypeptide (Ck-beta-8) and a method for treatment of a patient by administering Ck-beta-8 polypeptide.
- II. Claim 15, drawn to an agonist for Ck-beta-8 polypeptide.
- III. Claims 16 and 19, drawn to an antagonist for Ck-beta-8, and a method for treatment of a patient by administering the antagonist for Ck-beta-8 polypeptide.
- IV. Claim 18, drawn to a method for treatment of a patient by administering DNA encoding Ck-beta-8 polypeptide.
- V. Claim 20, drawn to a process for diagnosing a disease or susceptibility to a disease by determining a mutation in the nucleic acid sequence encoding the Ck-beta-8 polypeptide.
- VI. Claim 21, drawn to a diagnostic process for analyzing the presence of the Ck-beta-8 polypeptide.
- VII. Claim 22, drawn to a process for identifying antagonists and agonists of the Ck-beta-8 polypeptide.

The inventions listed as Groups I-VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-III are drawn to structurally different products which do not share the same or a corresponding special technical feature. Groups IV-VII are drawn to methods having different goals, method steps, and starting materials which do not share the same or a corresponding special technical feature which define the contribution of each invention. Since a special technical feature is not shared by each product, the inventions of Groups I-VIII do not form a single inventive concept within the meaning of Rule 13.2.